

Applicants: Corvalan et al.
U.S.S.N.: 10/041,860

Amendments to the Drawings:

The attached sheets of drawings includes changes to Figures 23-40. These sheets replace original sheets including Figures 23-40. The figures have been amended to recite SEQ ID NOs.

Attachments: Replacement Sheets, Figures 23-40.

Annotated Sheets showing changes.

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REMARKS

Claims 1, 2, and 22-45 are currently pending. Claims 22-23, 30, 32-33, 40 and 42-44 have been amended herein. All the amended claims are fully supported by the instant specification as originally filed. No new matter has been introduced.

Objections

The disclosure is objected to by the Examiner for allegedly failing to comply with the requirements of 37 CFR 1.821(d). According to the Examiner SEQ ID NOs are required for Figures 23-40. Replacement Figures 23-40 with SEQ ID NOs inserted have been provided under Express Mail Label EV490407343 US, mailing date November 19, 2004. An additional copy is provided herein. Also provided are annotated sheets showing the changes made to the figures.

Claims 2, 23, 33 and 44 are objected to as being dependent upon a rejected base claim. Applicants appreciate Examiner's finding of allowability for these claims if written in independent form. While not agreeing with the Examiner's rejections of the base claims (as addressed below), Applicants have amended claims 2, 23, 33 and 44 to be in independent form in order to merely expedite prosecution. As amended, claims 2, 23, 33 and 44 are allowable.

Rejections under 35 U.S.C. §112, first paragraph

Claims 1, 22, 24-32, 34-43 and 45 remain rejected by the Examiner under 35 U.S.C. §112, first paragraph, because the specification allegedly does not reasonably provide enablement for any human monoclonal antibody that binds to any Platelet Derived Growth Factor D, allegedly not enabling any person skilled in the art to which it pertains to make and use the invention commensurate in scope with the claims.

Legal Standard of Enablement:

To enable the claimed invention, the specification must describe to one skilled in the art, how to make and use the invention. With regards to the test of enablement, the MPEP states:

"Any analysis of whether a particular claim is supported by the disclosure in an application requires a determination of whether that disclosure, when filed, contained sufficient information regarding the subject matter of the claims as to enable one skilled in the pertinent art to make and use the claimed invention." (MPEP 2164.01)

For the Examiner to find a case of non-enablement, the Examiner must consider the following factors: (A) breadth of claims including:

- (i) How broad the claim is with respect to the disclosure? Applicants submit that the specification broadly discloses human antibodies to PDGFD including 19 specific embodiments. The pending claims are directed to human antibodies to PDGFD comprising specific sequences (claims 1, 2, 23-31, 33-39 and 44) or derived, or encoded by specific human antibody germline sequences (claims 22, 32, 42, 43 and 45). Pending claims are further supported by at least one specific embodiment. The scope of the claims is certainly within that disclosed by the specification; and
- (ii) Is one skilled in the art is enabled to make and use the entire scope of the claimed invention without undue experimentation? Applicants respectfully submit that the teachings of the present application carried out, successfully produced 19 human antibodies to PDGFD.

(B) nature of invention pertains to monoclonal antibodies, a technology that is relatively common, well known and widely practiced in the field of biotechnology; (C) state of the prior art for monoclonal antibodies is extensive and well established; (D) the level of one of ordinary skill in monoclonal antibody technology was determined *In re Wands* to be high and methods needed to practice a monoclonal antibody invention were well known; (E) level of predictability in the art is considered high as the U.S. PTO has issued claims to monoclonal antibodies for which no working examples are provided for in the specification as it is well established that monoclonal antibody technology is well known and generally considered reasonably predictable and routine; (F) the inventors have provided direction including specific methods of making the claimed human antibodies, the immunogen, and 19 specific embodiments and methods of using same; (G) existence of 19 specific working examples provided by the inventors; (H) quantity of experimentation needed to make or use invention is low as the invention is specifically described and supported by 19 working examples.

In order to make a rejection, the Examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph unless there is a reason to doubt the objective truth of the statements

contained therein which must be relied on for enabling support. It is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure. The minimal requirement is for the examiner to give reasons for the uncertainty of the enablement. Examiner should specifically identify what information is missing and why one skilled in the art could not supply the information without undue experimentation. Specific technical reasons are required. Applicants respectfully submit that a *prima facie* case of lack of enablement has not been made by Examiner.

Antibodies to PDGFD are enabled:

In response to Applicants' arguments filed November 19, 2004, the Examiner alleges:

"The specification merely discloses human PDGF-D as an immunogen for making the human monoclonal antibody. There is insufficient guidance about the structure of other PDGF-D other than human PDGF-D, much less about the specificity of the claimed antibody." (See OA page 7, lines 5-8)

Although Applicants agree that the antibodies of the present invention include antibodies that specifically bind non-human PDGFD, Applicants strongly disagree with the Examiners contention. Applicants provide the attached reference, Ostendorf, T. et al "*A Fully Human Monoclonal Antibody (CR002) Identifies PDGF-D as a Novel Mediator of Mesangioproliferative Glomerulonephritis*", J Am Soc Nephrol 14:2237-2247, 2003 ("Ostendorf" Exhibit A) in which it is shown that CR002.6.4 (which is Cur 2-6.4 of the current invention) binds specifically to human, murine and rat PDGFDD (DD meaning homodimer of PDGFD), see page 2242-2243 and Figure 4. The present specification therefore provides at least one specific embodiment with specificity to PDGFD, broader than merely human PDGFD, clearly enabling the claimed invention.

Combination of antibody heavy and light chain enabled:

The Examiner further alleges:

"With regard to claim 1, it is known that heavy and light chain combine to form antibody and it is the variable domains of the heavy and light chains form the antigen binding site. There is insufficient guidance as to the structure of the light chain without the amino acid sequence in the human monoclonal antibody as recited in claim 1." (See OA, page 7, lines 8-11.)

Applicants strongly disagree. The MPEP Eighth Edition, August 2001, Latest Revision May 2004 (2164.08) states:

"When analyzing the enabled scope of a claim, the teachings of the specification must not be ignored because claims are to be given their broadest reasonable interpretation that is consistent with the specification. "That claims are interpreted in light of the specification does not mean that everything in the specification must be read into the claims."

Raytheon Co. v. Roper Corp., 724 F.2d 951, 957, 220 USPQ 592, 597 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 835 (1984)."

Applicants respectfully submit that a person skilled in the art can clearly obtain guidance with respect to the structure of the light chain from the specification (see also, *W.L. Gore & Assoc., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1558, 220 USPQ 303, 316-17 (Fed. Cir. 1983); *In re Johnson*, 558 F.2d 1008, 1017, 194 USPQ 187, 195 (CCPA 1977)). For example, the specification specifically provides: 1) light chain germline sequences (see page 62, lines 18-2 and page 64, lines 1-24); 2) specific light chain sequences (Figures 3-21, nucleic acid sequences SEQ ID NOS: 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 81, 83, 85, 87, 89, 91, amino acid sequence SEQ ID NOS: 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 39, 41, 43, 45, 47, 49 and Figure 49); and 3) specific light chain CDR sequences for a human monoclonal antibody that binds to PDGFD (see Figure 49). The instant application specifically teaches that VH1-8 derived sequences can be combined with sequences derived from light chain germline sequences A19 (Antibody 1.49); A20 (Antibody 1.45); A27 (Antibody 6.4); and A30 (antibodies 1.19, 1.18 and 1.46). More specifically, for example, the specification teaches that light chain SEQ ID NO: 49 can be paired with the heavy chain of SEQ ID NO: 48, see Example 7 beginning at page 56 and Figure 21. Therefore the subject matter of claim 1 is fully enabled by the specification as filed.

V D J genes for antibodies specific for PDGFD are enabled:

The Examiner contends:

"The scope of claims 22 encompasses any human monoclonal antibody that binds to any PDGFD and said antibody is encoded or derived from any human VH1-8, any JH6B family gene and/or any D5-18 family gene. The specification on page 51 discloses only 13 antibodies to human PDGFD. The specification is silence whether the claimed antibodies binds to PDGFD other than human Platelet Derived Growth Factor D (PDGFD) encoded by the specific combination of human VH1-8 family gene and JH6B family gene. Without the nucleotide sequence of the family genes mentioned above, it is unpredictable which V sequence combine with which J sequence and/or D sequence to provide a human antibody that binds to any PDGFD. In contrast to applicant's assertion

that introns are irrelevant to enablement of the invention, the definition of a “gene” encompasses introns, exons and promoter regions.” (See OA page 7, lines 12-21.)

Applicants strongly disagree, however to more distinctly point out what Applicants regard as their invention, claims 22, 23, 42 and 44 (and claims 24-31 and 45, dependent thereon) recite or have been amended to recite “encoded by VH1-8 and JH6B”; claims 32 and 33 (and claims 34-39, 41, dependent thereon) recite or have been amended to recite “derived from VH1-8 and JH6B”; claim 40 has been amended to recite “derived from D5-18”; and claim 43 has been amended to recite “encoded by D5-18”. One of skill in the art would appreciate that the human VH1-8 gene is a specific sequence (not a collection of sequences) and is well known in the art and is specifically disclosed in the present specification at pages 62-63. Furthermore, one of skill in the art would know that the human JH6B gene is a specific sequence and is well known in the art and is specifically described by Rabbits, T. H. (1983). Biochem. Soc. Trans., 11, 119-126 (Exhibit B, attached hereto). Additionally, one of skill in the art would know the human D5-18 gene is a specific sequence which is well known in the art and is specifically described by Corbett et al (1997). J. Mol. Biol., 270, 587-597 (Exhibit C, attached hereto). As stated in the MPEP 2164.05(a):

“the specification need not disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), cert. denied, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).”

Accordingly, Applicants need not provide the nucleotide sequence of human VH1-8, JH6B and D5-18, for the present invention to be enabled as this information is readily available in the public domain and one of skill in the art would know how to obtain such information. Furthermore, one of skill in the art would be able to obtain any needed information regarding introns, exons and promoter regions from the public domain. Homologs of human VH1-8, JH6B and D5-18 genes in other species are not within the scope of human monoclonal antibodies of the invention. Furthermore, as stated above, the antibodies of the invention do bind to PDGFD other than human PDGFD. The scope of claims 22, 23 and dependent claims 24-31, claims 32, 33 and dependent claims 34-39, 41, claims 42, 44 and dependent claim 45 encompasses antibodies

that specifically bind to PDGFD, wherein such antibodies are encoded by human VH1-8 and JH6B and is fully enabled by the present specification as filed.

The Examiner seems to contend that providing the nucleotide sequence of human VH1-8 family gene, a JH6B family gene and a human D5-18 family gene is necessary to avoid unpredictability of which V D J sequences combine to provide a human antibody that binds to PDGFD. The present specification provides *extensive* specific examples for the following examples of V D J combinations which provide human antibodies specific to PDGFD:

VH1-8 with D2 and JH6b (exemplified by Antibodies 1.40.1 and 1.46.1);
VH1-8 with D3-16 and JH6b (exemplified by Antibody 1.19.1);
VH1-8 with D5-12 and JH6b (exemplified by Antibody 1.49.1);
VH1-8 with D5-18 and JH6b (exemplified by Antibody 6.4.1);
VH1-8 with D6-19 and JH6b (exemplified by Antibody 1.18);
VH1-8 with DK4 and JH6b (exemplified by Antibody 1.45);
VH1-18 with D21-9 and JH6b (exemplified by Antibody 1.33);
VH1-18 with D21-9 and JH4b (exemplified by Antibody 1.48.1);
VH3-21 with D3-16 and JH4b (exemplified by Antibody 1.6.1);
VH3-33 with D21-9 and JH6b (exemplified by Antibody 1.38.1);
VH3-33 with D5-18 and JH6b (exemplified by Antibodies 1.17.1 and 1.24.1);
VH3-53 with D4-17 and JH6b (exemplified by Antibody 1.11.1);
VH5-51 with D3-10 and JH4b (exemplified by Antibodies 1.23.1, 1.25.1 and 1.39.1);
VH5-51 with D3-16 and JH5b (exemplified by Antibody 1.51.1); and
VH5-51 with D5-12 and JH6b (exemplified by Antibody 1.29). See, for example, Figure

22A. Applicants have provided 7 antibodies that are encoded by or derived from VH1-8; 2 antibodies from VH1-18; 1 antibody from VH3-21; 3 antibodies from VH3-33; and 5 antibodies from VH5-51; all of which bind to PDGFD. Two antibodies are provided that are encoded by or derived from D2; 3 antibodies from D21-9; 3 antibodies from D3-10; 3 antibodies from D3-16; 1 antibody from D4-17; 2 antibodies from D5-12; 3 antibodies from D5-18; 1 antibody from D6-19; and 1 antibody from DK4; all of which bind to PDGFD. Five antibodies are provided that are encoded by or derived from JH4b; 1 antibody from JH5b; and 13 antibodies from JH6b, all of which bind to PDGFD. One of skill in the art, provided with the teachings of the present

specification would not find determining which V D J sequences to combine to make antibodies binding to PDGFD unpredictable but fully enabled.

The MPEP (2164.08) states:

“Claims are not rejected as broader than the enabling disclosure for noninclusion of limitations dealing with factors which must be presumed to be within the level of ordinary skill in the art; claims need not recite such factors where one of ordinary skill in the art to whom the specification and claims are directed would consider them obvious.”

Given the teachings of the present specification, one of skill in the art would be able to obtain VH1-8, JH6B and D5-18 genes, or to use a system known to have those genes (such as the XenoMouse™ animals described in the present specification, see pages 46-48 and Example 1) to generate the antibodies of the present invention.

Antibody CDRs are enabled:

The Examiner further alleges:

“In response to Applicants’ argument the antibody, in most cases will also have other CDR regions, framework regions as well as optionally constant domains. Therefore the open ended language “comprising” in claims 24-29 and 34-39 correctly reflects the invention, it is correct that antibody contains CDR1-3 from heavy and light chains, framework regions as well as heavy chain, and CDR1, CDR2 and CDR3 from the light chain, for example. Claim 25 as written lacks the CDR1 and CDR3 from the heavy chain, and CDR1-3 from the light chain. Claim 26 as written lacks the CDR1-2 from heavy chain and CDR1-3 from the light chain. Claim 27 as written lacks the CDR1-3 from the heavy chain and CDR2-3 from the light chain. Claim 28 as written lacks the CDR 1-3 from the heavy chain and CDR1 and CDR3 from the light chain. Likewise claims 29 lacks the CFR1-3 from the heavy chain and CDR1-2 from the light chain. The same reasons apply to claims 34-39. There is insufficient guidance as to the undisclosed CDRs mentioned in said claims without the specified amino acid sequence.” (See OA page 7, lines 25-32 to page 8, line 5.)

In response, Applicant strongly disagrees with the Examiner’s position. The MPEP (2164.08) clearly states:

“One does not look to the claims but to the specification to find out how to practice the claimed invention.”

With regards to Claim 25, Applicants submit that examples of appropriate heavy chain CDR1 and CDR3 sequences and light chain CDR1, 2, 3 sequences are delineated in the specification, for example in Figure 48 (heavy chain) and Figure 49 (light chain). Similarly the CDRs the

Examiner alleges are lacking to enable claims 26, 27, 28 and 29 can be found, for example, in these same Figures in the specification as filed. Given a heavy chain or light chain sequence and a specific CDR sequence, multiple possible other CDR sequences are taught in the present specification. For example, claim 24 specifies a CDR1: GYTFTSYDIN. This CDR1 can be combined with a CDR2 of WMNPNSGNTGYAQKFQGR (see for example antibody 1.19) or WINPNSGNTDYAQKFQGR (see for example antibody 6.4) or WMNPNNNGNTGYAQKFQGR (see for example antibody 1.46) or WMNPNSGDTGYAQKFQGR (see for example antibody 1.49) or WISAYNGNTNYAQKLQGR (see for example antibody 1.33). Therefore, these claims are clearly enabled by the specification as filed.

Applicants respectfully submit that the pending claims are fully enabled by the present specification and a *prima facie* case of lack of enablement has not been made by Examiner. Applicants respectfully request that this rejection be withdrawn.

Rejections under 35 U.S.C. §112, first paragraph

The Examiner has rejected claims 1-2, 22, 24-32, 34-43 and 45 under 35 U.S.C. §112, first paragraph as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention.

Applicant respectfully strongly disagrees. The first paragraph of 35 U.S.C. 112 requires that the "specification shall contain a written description of the invention. To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. See, e.g., *Moba, B.V. v. Diamond Automation, Inc.*, 325 F.3d 1306, 1319, 66 USPQ2d 1429, 1438 (Fed. Cir. 2003); *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d at 1563, 19 USPQ2d at 1116."

Claim 1 is to a human monoclonal antibody specific for PDGFD comprising SEQ ID NO:48 and claim 2 further comprises SEQ ID NO: 49. Applicant clearly had possession of this invention at the time the application was filed as evidenced for example by Example 7, page 56-69, specifically human monoclonal antibody Cur 2 6.4, see Figure 21.

Claim 22, 32, 42 and are to a human monoclonal antibody specific for PDGFD encoded by, or derived from germline VH1-8 and JH6b. Claim 23, 33, and 44 further comprises SEQ ID NO: 48 and 49. Applicant respectfully points out that antibodies 1.40.1, 1.49.1, 1.45, 6.4.1, 1.19.1, 1.18, and 1.46.1 (Example 7 and Figures 15, 19, 16, 21, 7 6, 17) are encoded by VH1-8 and JH6b as described in the specification and that antibody Cur 2 6.4 further comprises SEQ ID NO:48 and 49 (Figure 21). Therefore Applicants were in possession of this invention at the time the application was filed. Dependent claims 24-31 and 34-41, 43, and 45 are also therefore supported by the present written description.

Sufficient written description of the light chain is provided:

In response to Applicants' arguments filed November 19, 2004, the Examiner alleges:

"Other than the specific human monoclonal antibody that binds to human PDGF-D comprising the specific combinations of heavy and light chains as set forth in claim 23, there is insufficient written description about the structure of the light chain in the monoclonal antibody in claim 1 without the amino acid sequence." (See OA starting at page 10, last paragraph.)

Applicants respectfully submit that this is clearly incorrect. The specification unquestionably provides written description as to the structure of the light chain. The specification specifically provides: 1) light chain germline sequences (see page 62, lines 18-2 and page 64, lines 1-24); 2) specific light chain sequences (Figures 3-21, nucleic acid sequences SEQ ID NOs: 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 81, 83, 85, 87, 89, 91, amino acid sequence SEQ ID NOs:14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 39, 41, 43, 45, 47, 49 and Figure 49); and 3) specific light chain CDR sequences for a human monoclonal antibody that binds to PDGFD (see Figure 49). Applicants have specifically provided written description that VH1-8 derived sequences can be combined with sequences derived from light chain sequences A19 (Antibody 1.49); A20 (Antibody 1.45); A27 (Antibody 6.4); and A30 (antibodies 1.19, 1.18 and 1.46) to obtain antibodies specific for PDGFD. More specifically, for example, Applicants described one possible light chain that can be paired with the heavy chain of SEQ ID NO: 48 is light chain SEQ ID NO: 49, see Example 7 beginning at page 56, Figure 21. The present specification contains written description of 19 specific physical embodiments of a human monoclonal antibody that binds to PDGFD.

Sufficient written description of PDGFD is provided:

The Examiner contends:

“Further the specification discloses only human monoclonal antibody or antigen binding portion thereof that specifically binds to human PDGF-D. There is a lack of written description about the other PDGF-D to which the claimed antibody binds.” (See OA, page 11, lines 2-5.)

Applicants disagrees. The present specification described and discloses human monoclonal antibodies binding PDGFD. As described above, the antibodies of the invention do bind PDGF-D other than *human* PDGFD. The written description of these antibodies, for example, Cur 6.4, (Example 7 and Figure 21) therefore provides adequate written description of antibodies that bind to PDGFD other than human PDGFD.

Sufficient written description of VH1-8, JH6B and D5-18 is provided:

The Examiner states:

“With regard to claims 22, 30, 32 and 42, the family of genes such as human “VH1-8 family gene”, any “JH6B family gene” and/or human “D5-18 family gene” as recited in claims 22, 30, 32 and 42-43 without the nucleotide sequence have no structure much less function. The specification provides insufficient written description about which particular VH1-8 family gene and JH6B family gene encode the claimed antibody or which undisclosed human monoclonal antibody that binds to all PGDF-D is derived from which particular human VH1-8 family gene and JH6B family gene encode the claimed antibody or which undisclosed human monoclonal antibody that binds to all PGDF-D is derived from which particular human VH1-8 family gene and JH6B family gene without the nucleotide sequence (claims 22, 32 and 42).” (See OA, page 11, lines 6-12.)

Applicants disagree. Applicants have shown that VH1-8, JH6B and D5-18 sequences and genes themselves are well known to one of skill in the art. Applicants needn’t disclose or describe that which is known in the art. Applicants respectfully point out that there is one human VH1-8 gene and one JH6B gene as is well understood in the art, rendering the Examiner’s written description rejection based upon lack of guidance as to “*which particular* VH1-8 family gene and JH6b family gene encode the claimed antibody...” moot. To clarify, claims 22, 23, 42 and 44 (and claims 24-31 and 45 dependent thereon) recite or have been amended to recite “encoded by VH1-8 gene and JH6B gene”; claims 32 and 33 (and claims 34-39, 41 dependent thereon) recite or have been amended to recite “derived from VH1-8 and JH6B”; claim 40 has been amended to recite “derived from D5-18”; and claim 43 has been amended to recite “encoded by D5-18 gene”.

Sufficient written description of structure of claimed antibody is provided:

The Examiner contends:

“The specification provides insufficient written description about the introns as well as exons in the germ line that encodes the undisclosed human monoclonal antibody, let alone a gene from the human VH1-8 and JH6B gene that encode any human monoclonal antibody or antigen binding portion thereof that specifically binds to all Platelet Derived Factor D (PDGF-D). Since the structure of the claimed antibody mentioned above are not adequately described, it follows that any human monoclonal antibody further comprises a detectable marker are not adequately described. It also follows that any composition comprising any human monoclonal antibody mentioned above are not adequately described.” (See OA, page 11, lines 15-23.)

Applicants respectfully submit that the nucleotide sequences of known VH1-8, JH6B and D5-18 genes, and information regarding introns, exons and promoter regions pertaining to these genes is known in the art and therefore one of skill in the art would be able to obtain the information if needed. Furthermore, such introns, exons and promoter regions are not part of the *structure* of the claimed human monoclonal antibodies. The structure of a monoclonal antibody in general, is exceptionally well known in the art, and is described in the specification (see pages 34-35). Furthermore the pending claims detail such structure by including specific sequence and germline lineage limitations. The claimed antibodies are supported by the written description provided in the present specification.

Sufficient written description of CDRs of claimed antibody is provided:

The Examiner alleges:

“...claim 24 as written lacks the CDR2 and CDR3 from the heavy chain, and CDR1, CDR2 and CDR3 from the light chain...There is inadequate written description about the other undisclosed CDRs mentioned above in said claims without the specified amino acid sequence.” (See OA page 11, line 28 to page 12, line 4.)

Applicants disagree. For example, with regards to Claim 25, which the Examiner contends lacks written description for the CDR1 and CDR3 from the heavy chain, and CDR1-3 from the light chain, Applicants submit that examples of appropriate heavy chain CDR1 and CDR3 sequences and light chain CDR1, 2, 3 sequences are delineated for example in Figure 48 (heavy chain) and Figure 49 (light chain) in the specification as filed. Similarly the CDRs the Examiner alleges is lacking written description for claims 26, 27, 28 and 29 can be found, for example, in these same Figures in the specification as filed. Therefore, these claims are clearly supported by sufficient written description provided in specification as filed.

The Examiner has the initial burden, after a thorough reading and evaluation of the content of the application, of presenting evidence or reasons why a person skilled in the art would not recognize that the written description of the invention provides support for the claims. There is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed, *Wertheim*, 541 F.2d at 262, 191 USPQ at 96.

Applicants respectfully submit that the claimed invention is well supported by sufficient written description provided in the present specification. Applicants request that this rejection be withdrawn.

Rejections under 35 U.S.C. §103(a)

Claims 22, 30-32, 40-43 and 45 remain rejected by the Examiner under 35 U.S.C. §103(a) as being unpatentable over US Patent 6, 706, 687 in view of Green et al. The Examiner asserts that:

“The nucleotide sequences of “VH1-8”, VH6B”(sic) and “D5-18” that encode the claimed human antibody that binds to human PDGF-D are not recited in the claims. In the absence of the specific nucleotide sequences of “VH1-8”, VH6B” and “D5-18”, the process of recombination in the method as taught by Green would produce the claimed invention.” (See OA page 14, lines 15-18.)

Applicants respectfully disagree. The method taught by Green did not teach or suggest a human monoclonal antibody derived from VH1-8, JH6B (and furthermore from D5-18) which would have specificity to PDGFD. Nor could the teachings of Green have made it obvious which V, J or D genes from amongst the various gene segments available (45 heavy chain V; 35 kappa V; 23 heavy chain D; 6 heavy chain J; 5 kappa J) to combine to obtain specificity for PDGFD. Until the applicants’ invention was made, it could not have been known to specifically use VH1-8, VH6B (and furthermore, D5-18) to provide for a human antibody that binds to PDGFD.

Applicants respectfully request that this rejection be withdrawn.

CONCLUSION

Applicant respectfully requests that the amendments and remarks made herein be entered and made of record in the file history of the present application. Applicant respectfully submits that this paper is fully responsive and that the pending claims are in condition for allowance.

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U.S.S.N.: 10/041,860

Such action is respectfully requested. If there are any questions regarding these amendments and remarks, the Examiner is encouraged to contact the undersigned at the telephone number provided below.

Respectfully submitted,



Date: May 2, 2005

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ANTIBODIES DIRECTED TO PDGFD AND USES THEREOF
Corvalan et al.
 Appl. No.: 10/041,860. Atty Docket: ABGENIX.051A

FIGURE 23

Figure 23A

										Section 1																																						
										10	20	30	40	51																																		
CUR2-1.6.1 HC										(1) EVQLVERGGGLVKPGGLRLSCAASGP	Y	M	N	W	Q	R	G	L	E	W	V	S	I																									
VH3-21										(1) EVQLVERGGGLVKPGGLRLSCAASGP	T	F	S	M	N	W	R	A	G	G	L	E	W	V	S	I																						
Consensus										(1) EVQLVERGGGLVKPGGLRLSCAASGP	P	S	Y	M	N	W	R	A	P	G	R	G	E	W	V	S	I																					
										52	60	70	80	90	102	Section 2																																
CUR2-1.6.1 HC										(52) S	S	S	S	S	Y	Y	A	D	S	V	K	G	R	P	T	I	S	R	D	N	A	K	N	S	L	R	A	E	D	T	A	V	Y	C	A	R	I	M
VH3-21										(52) S	S	S	S	S	Y	Y	A	D	S	V	K	G	R	P	T	I	S	R	D	N	A	K	N	S	L	R	A	E	D	T	A	V	Y	C	A	R	I	M
Consensus										(52) S	S	S	S	S	Y	Y	A	D	S	V	K	G	R	P	T	I	S	R	D	N	A	K	N	S	L	R	A	E	D	T	A	V	Y	C	A	R	I	M
										103	110	126						Section 3																														
CUR2-1.6.1 HC										(103) T	F	G	G	I	I	A	S	F	Y	D	Y	W	G	G	T	L	V	S	SEQ ID NO:13						Section 1													
VH3-21										(99) -----						SEQ ID NO:3						Section 2																										
Consensus										(103)																Section 3																						

SEQ ID NOS have been added

Figure 23B

										Section 1																																		
										10	20	30	40	51																														
CUR2-1.6.1 LC										(1) D	I	Q	Q	K	P	G	K	A	P	K	R	L	I	Y	A																			
A30										(1) D	I	Q	Q	K	P	G	K	A	P	K	R	L	I	Y	A																			
Consensus										(1) D	I	Q	Q	K	P	G	K	A	P	K	R	L	I	Y	A																			
										52	60	70	80	90	102	Section 2																												
CUR2-1.6.1 LC										(52) S	S	L	Q	S	G	V	P	S	R	F	3	G	3	G	5	G	T	E	F	T	I	T	S	I	S	L	Q	H	N	S	Y	P	-----	Section 1
A30										(52) S	S	L	Q	S	G	V	P	S	R	F	3	G	3	G	5	G	T	E	F	T	I	T	S	I	S	L	Q	H	N	S	Y	P	-----	Section 2
Consensus										(52) S	S	L	Q	S	G	V	P	S	R	F	3	G	3	G	5	G	T	E	F	T	I	T	S	I	S	L	Q	H	N	S	Y	P	-----	Section 3
										103	107						Section 3																											
CUR2-1.6.1 LC										(103) K	V	E	I	K	SEQ ID NO:14						Section 1																							
A30										(96) -----	SEQ ID NO:11						Section 2																											
Consensus										(103)											Section 3																							

SEQ ID NOS have been added

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FIGURE 24

Figure 24A

	1	10	20	30	40	51	Section 1
Cur2-1.11.1 HC	(1)						
VH3-53	(1)	EVQLVESGGGLIQPGGSLRLSCAASGPTVSSNYMSWVRQAPGKGLIEWVSVI					
Consensus	(1)	EVQLVESGGGLIQPGGSLRLSCAASGPTVSSNYMSWVRQAPGKGLIEWVSVI					Section 2
	(52)	52	60	70	80	90	102
Cur2-1.11.1 HC	(52)	YSGGSTYYADSVKGRFTIQRDN SKNTLYLOMNSIPLAEDTAVYYCAGTVTTN					
VH3-53	(52)	YSGGSTYYADSVKGRFTIQRDN SKNTLYLOMNSIPLAEDTAVYYCAGTVTTN					
Consensus	(52)	YSGGSTYYADSVKGRFTIQRDN SKNTLYLOMNSIPLAEDTAVYYCAGTVTTN					Section 3
	(103)	103	110	120			
Cur2-1.11.1 HC	(103)	YYYGMDVWGQGTTVTVSS			SEQ ID NO:15		
VH3-53	(98)				SEQ ID NO:5		
Consensus	(103)						

SEQ ID NOS
have been added

Figure 24B

	1	10	20	30	40	51	Section 1
CUR2-1.11.1 LC	(1)						
A19	(1)	DIVMTQSPLSLPVTPGEPAISCR8SQSLL					
Consensus	(1)	DIVMTQSPLSLPVTPGEPAISCR8SQSLL					Section 2
	(52)	52	60	70	80	90	102
CUR2-1.11.1 LC	(52)	LIYLGSNRASGVEDRPSGSGCTIPLKISRVEAE					
A19	(52)	LIYLGSNRASGVEDRPSGSGCTIPLKISRVEAE					
Consensus	(52)	LIYLGSNRASGVEDRPSGSGCTIPLKISRVEAE					Section 3
	(103)	103	111				
CUR2-1.11.1 LC	(103)	GGGTTKVEIK		SEQ ID NO:16			
A19	(101)			SEQ ID NO:8			
Consensus	(103)						

SEQ ID NOS
have been added

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FIGURE 25

Figure 25A

	1	10	20	30	40	51	Section 1
CR2-1.17.1 HC	(1)						
VH3-33		QVQIMPEGGGVVQPG	BLR1SCAASGFTPSSYGMHWVROAPGKGLEWVAVI				
Consensus		(1)	QVQIMPEGGGVVQPG	BLR1SCAASGFTPSSYGMHWVROAPGKGLEWVAVI			
		(1)	QVQIMPEGGGVVQPG	BLR1SCAASGFTPSSYGMHWVROAPGKGLEWVAVI			
							Section 2
	(52)	52	60	70	80	90	102
CR2-1.17.1 HC	(52)	WYDGSNKKYADSVKGRPTISRDNSKNTLYLQMN	QMN	SLRAEDTAVVYCARDOGY			
VH3-33	(52)	WYDGSNKKYADSVKGRPTISRDNSKNTLYLQMN	QMN	SLRAEDTAVVYCAR			
Consensus	(52)	WYDGSNKKYADSVKGRPTISRDNSKNTLYLQMN	QMN	SLRAEDTAVVYCAR			
							Section 3
	(103)	103	110		126		
CR2-1.17.1 HC	(103)	RYAGYYYDYGMDVWGQTTTVVSS	SEQ ID NO:17				
VH3-33	(99)	-----	-----	SEQ ID NO:4			
Consensus	(103)						

Figure 25B

	1	10	20	30	40	52	Section 1
CR2-1.17.1 LC	(1)						
A30		DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKR	LIYAS				
Consensus	(1)	DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKR	LIYAS				
		(1)	DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKR	LIYAS			
							Section 2
	(53)	53	60	70	80	90	104
CR2-1.17.1 LC	(53)	SIQSGVPSPFSGSGSGTEFTLTISSLQPEDFATYCYCLOHNSYPLTF	GGGTRV				
A30	(53)	SIQSGVPSPFSGSGSGTEFTLTISSLQPEDFATYCYCLOHNSYPLTF	GGGTRV				
Consensus	(53)	SIQSGVPSPFSGSGSGTEFTLTISSLQPEDFATYCYCLOHNSYPLTF	GGGTRV				
							Section 3
	(105)	105					
CR2-1.17.1 LC	(105)	EIK	SEQ ID NO:18				
A30	(96)	-----	SEQ ID NO:11				
Consensus	(105)						

SEQ ID NOS
 have been added

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FIGURE 26

Figure 26A

						Section 1
	(1)	10	20	30	40	52
CR2-1.18 HC	(1)	QVQLVQSGAEVKKPGASVVKVSCKASGYTFTSYDINWVRQATGQGLEWMGWMN				
VH1-8	(1)	QVQLVQSGAEVKKPGASVVKVSCKASGYTFTSYDINWVRQATGQGLEWMGWMN				
Consensus	(1)	QVQLVQSGAEVKKPGASVVKVSCKASGYTFTSYDINWVRQATGQGLEWMGWMN				
	(53)	60	70	80	90	104
CR2-1.18 HC	(53)	PNESGNTGYAQKFQGRVTMTRNTSISTAYMELSLRSEDTAVYYCAR				
VH1-8	(53)	PNESGNTGYAQKFQGRVTMTRNTSISTAYMELSLRSEDTAVYYCAR				
Consensus	(53)	PNESGNTGYAQKFQGRVTMTRNTSISTAYMELSLRSEDTAVYYCAR				
						Section 2
	(105)	110	126			
CR2-1.18 HC	(105)	GTYYYYYYGMDVWGQGT	TVTVSS			
VH1-8	(99)	-----				
Consensus	(105)	-----				

SEQ ID NO:19
SEQ ID NO:1

Figure 26B

						Section 1
	(1)	10	20	30	40	53
CR2-1.18 LC	(1)	DIQMTQSPSSLSASVGDRVTITCRASQGIRNDIGWYQQKPGKAPKR	LIYAA			
A30	(1)	DIQMTQSPSSLSASVGDRVTITCRASQGIRNDIGWYQQKPGKAPKR	LIYAA			
Consensus	(1)	DIQMTQSPSSLSASVGDRVTITCRASQGIRNDIGWYQQKPGKAPKR	LIYAA			
	(54)	60	70	80	90	106
CR2-1.18 LC	(54)	IQSGVPSRPGSGSGTEFTLTISLQPEDPATYFCLOHN	SYP			
A30	(54)	IQSGVPSRPGSGSGTEFTLTISLQPEDPATYFCLOHN	SYP			
Consensus	(54)	IQSGVPSRPGSGSGTEFTLTISLQPEDPATYFCLOHN	SYP			
						Section 2
	(107)	R				
CR2-1.18 LC	(107)	SEQ ID NO:20				
A30	(96)	SEQ ID NO:11				
Consensus	(107)	-----				

SEQ ID NOS
have been added

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FIGURE 27

Figure 27A

											Section 1
	(1)	1	10	20	30	40	50	52			
Cur2-1.19.1 hc	(1)	QVOLVQSGAEVKREGASVKSCKASGYTFTSYDINWVRQATGQGLEWMGMN									
VH1-8	(1)	QVOLVQSGAEVKREGASVKSCKASGYTFTSYDINWVRQATGQGLEWMGMN									
Consensus	(1)	QVOLVQSGAEVKREGASVKSCKASGYTFTSYDINWVRQATGQGLEWMGMN									
											Section 2
	(53)	53	60	70	80	90		104			
Cur2-1.19.1 hc	(53)	PNSGNTGYAQKPFQGRVTMTRNTSISTAYMELSSLRSEDTAVYYCAR									
VH1-8	(53)	PNSGNTGYAQKPFQGRVTMTRNTSISTAYMELSSLRSEDTAVYYCAR									
Consensus	(53)	PNSGNTGYAQKPFQGRVTMTRNTSISTAYMELSSLRSEDTAVYYCAR									
											Section 3
	(105)	105	110	120							
Cur2-1.19.1 hc	(105)	GGVIVHYGMDVWGQGTTTVVSS									
VH1-8	(99)	-----									
Consensus	(105)	-----									

SEQ ID NO:21
 SEQ ID NO:1

Figure 27B

											Section 1
	(1)	1	10	20	30	40	50	52			
Cur2-1.19.1 lc	(1)	DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGRAFRLIYAS									
A30	(1)	DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGRAFRLIYAS									
Consensus	(1)	DIQMTQSPSSLSASVGDRVTITCRASQGIRNDLGWYQQKPGRAFRLIYAS									
											Section 2
	(53)	53	60	70	80	90		104			
Cur2-1.19.1 lc	(53)	SIQSGVPSPRSCSGSGTDFTLTISLQPEDATYYCLQHNSP									
A30	(53)	SIQSGVPSPRSCSGSGTDFTLTISLQPEDATYYCLQHNSP									
Consensus	(53)	SIQSGVPSPRSCSGSGTDFTLTISLQPEDATYYCLQHNSP									
											Section 3
	(105)	1087									
Cur2-1.19.1 lc	(105)	EIR									
A30	(96)	-----									
Consensus	(105)	-----									

SEQ ID NO:22
 SEQ ID NO:11

SEQ ID NOS
 have been added

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FIGURE 28

Figure 28A

	1	10	20	30	40	51
Cur2-1.23.1 HC VH5-51	(1)					
Consensus	(1)	EVQLVQSGAEVKPGESLKISC	GGYSPTSYWIGWVRQMPGKGLEWMGII			
						Section 1
	52	60	70	80	90	102
Cur2-1.23.1 HC VH5-51	(52)	YPGDSDTRYSPSPFGQVTISADKSISTAYLQWSLRA	SDTAMYYCARHVSY			
Consensus	(52)	YPGDSDTRYSPSPFGQVTISADKSISTAYLQWSLRA	SDTAMYYCARHVSY			
						Section 2
	103	110	126			
Cur2-1.23.1 HC VH5-51	(103)	YVVS	SGSYNNVPDYWGQGT	SEQ ID NO:23		
Consensus	(103)	YVVS	SGSYNNVPDYWGQGT	SEQ ID NO:6		
						Section 3

Figure 28B

	1	10	20	30	40	51
Cur2-1.23.1, LC A30	(1)					
Consensus	(1)	DIQMTQSPSLSASAVGDRVTITCRASQ	IRNDLGWYQQIPGKAPKR	LIYAA		
		DIQMTQSPSLSASAVGDRVTITCRASQ	IRNDLGWYQQIPGKAPKR	LIYAA		Section 1
	52	60	70	80	90	102
Cur2-1.23.1, LC A30	(52)	SSLQGVPSRFPSSGSGSGTEFTLT	ISLQPEDPATYYCLQHNSYP			
Consensus	(52)	SSLQGVPSRFPSSGSGSGTEFTLT	ISLQPEDPATYYCLQHNSYP			
						Section 2
	103	107				
Cur2-1.23.1, LC A30	(103)	KVEIK	SEQ ID NO:24			
Consensus	(103)	KVEIK	SEQ ID NO:11			
						Section 3

SEQ ID NOS
 have been added

FIGURE 29

Figure 29A

		Section 1				
		10	20	30	40	50
CR2-1.24.1_HC VH-33	(1)	QVQLVE	GGGVVQPGRSI	RLSCAASG	FSSYGMHWVRQAPGK	GLEWVADI
	(1)	QVQLVE	GGGVVQPGRSI	RLSCAASG	FSSYGMHWVRQAPGK	GLEWVAVI
	Consensus	(1)	QVQLVE	GGGVVQPGRSI	RLSCAASG	PSSYGMHWVRQAPGK
		Section 2				
CR2-1.24.1_HC VH-33	(52)	52	60	70	80	90
	(52)	WYDGS	NKYYADSVKGRFTI	SRDN	S	KNTLYLQMNSLRAE
	(52)	WYDGS	NKYYADSVKGRFTI	SRDN	S	KNTLYLQMNSLRAE
Consensus	(52)	WYDGS	NKYYADSVKGRFTI	SRDN	S	KNTLYLQMNSLRAE
	(52)	WYDGS	NKYYADSVKGRFTI	SRDN	S	KNTLYLQMNSLRAE
		Section 3				
CR2-1.24.1_HC VH-33	(103)	103	110	126		
	(103)	SYGYV	YYDYGMDVWQGTTVTVSS		SEQ ID NO:25	
	(99)	-	-		SEQ ID NO:4	
Consensus	(103)					

Figure 29B

SEQ ID NOS
have been added

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FIGURE 30

Figure 30A

	1	10	20	30	40	51	Section 1
VH5-51	(1)						
CR2-1.25.1_HC	(1)	EVOLVQSGAEVKKPGESLRKISCKGSGYFTSYWIGWVRQMPGKGLEWMGII					
Consensus	(1)	EVOLVQSGAEVKKPGESLRKISCKGSGYFTSYWIGWVRQMPGKGLEWMGII					
	52	60	70	80	90	102	Section 2
VH5-51	(52)	YPGDSDTRYBPSPQGVITISADKSISTAYLQWSSLKASDTAMYYCAR					---
CR2-1.25.1_HC	(52)	YPGDSDTRYBPSPQGVITISADKSISTAYLQWSSLKASDTAMYYCARHGSY					
Consensus	(52)	YPGDSDTRYBPSPQGVITISADKSISTAYLQWSSLKASDTAMYYCAR					Section 3
	103	110	126				
VH5-51	(103)						
CR2-1.25.1_HC	(103)	YYGSETYYNVFDYWGQGTLVTVSS					
Consensus	(103)						

SEQ ID NO:6
SEQ ID NO:27

Figure 30B

	1	10	20	30	40	52	Section 1
A30	(1)						
CR2-1.25.1_LC	(1)	DIQMTQSPSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLLIYAS					
Consensus	(1)	DIQMTQSPSLSASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLLIYAS					
	53	60	70	80	90	104	Section 2
A30	(53)	SLQSGVPSRPSGSGSGTEFTLTISLQPEDFATYYCLQHNSYP					---
CR2-1.25.1_LC	(53)	SLQSGVPSRPSGSGSGTEFTLTISLQPEDFATYYCLQHNSYPWTFGQGTV					
Consensus	(53)	SLQSGVPSRPSGSGSGTEFTLTISLQPEDFATYYCLQHNSYP					Section 3
	105	1087					
A30	(96)	---					
CR2-1.25.1_LC	(105)	EIR					
Consensus	(105)						

SEQ ID NO:11
SEQ ID NO:28

SEQ ID NOS
have been added

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FIGURE 31

Figure 31A

	1	10	20	30	40	50	Section 1
VH5-51	(1)						
CR2-1.29_HC	(1)	EVQLVQSGABVKRPGE 31KISCKGSGY SPFNSWIGVROMPGKGL EWMGIIY					
Consensus	(1)	EVQLVQSGARVKRPGE 31KISCKGSGY SPFNSWIGVROMPGKGL EWMGIIY					
	(1)	EVQLVQSGAEVKRPGE 31KISCKGSGY SFTSYWIGVROMPGKGL EWMGIIY					
	53	60	70	80	90	104	Section 2
VH5-51	(53)	PGDSDTRYSPFQGQVALSADRKISTAYLQWSSLRA SDTAMYYCAR					
CR2-1.29_HC	(53)	PGDSDTRYSPFQGQVALSADRKISTAYLQWSSLRA SDTAMYYCAR					
Consensus	(53)	PGDSDTRYSPFQGQ TISADKSISTAYLQWSSLRA SDTAMYYCAR					
	105	110	129				Section 3
VH5-51	(105)	(99)					
CR2-1.29_HC	(105)	TIGGYYYYYYHGMDVWGQGTTVSS					
Consensus	(105)						

SEQ ID NO:6
SEQ ID NO:29

Figure 31B

	1	10	20	30	40	50	Section 1
A19	(1)						
CR2-1.29_LC	(1)	DIVMTOSPLSLPVTGPEASISCRSSQSLHSNGNYLDWYLQKPGQSPQLI					
Consensus	(1)	DIVMTQSP 1 LPVTGPEASISCRSSQSLHSNGNYLDWYLQKPGQSPQLI					
	(1)	DIVMTQSP 1 LPVTGPEASISCRSSQSLHSNGNYLDWYLQKPGQSPQLI					
	54	60	70	80	90	106	Section 2
A19	(54)	YLGSNRASGV 1 PD 1 FG 1 GT 1 DK 1 TR 1 SR 1 VEAD 1 DVG 1 Y 1 CM 1 Q 1 ALQS					
CR2-1.29_LC	(54)	YLGSNRASGV 1 PD 1 FG 1 GT 1 DK 1 TR 1 SR 1 VEAD 1 DVG 1 Y 1 CM 1 Q 1 ALQS					
Consensus	(54)	YLGSNRASGV 1 PD 1 FG 1 GT 1 DK 1 TR 1 SR 1 VEAD 1 DVG 1 Y 1 CM 1 Q 1 ALQS					
	107	113					Section 3
A19	(107)	(101)					
CR2-1.29_LC	(107)	GTKLEIK					
Consensus	(107)						

SEQ ID NO:8
SEQ ID NO:30

SEQ ID NOS
have been added

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FIGURE 32

Figure 32A

										Section 1
										52
VH1-18	(1)	1	10	20	30	40				
CR2-1.33_HC	(1)	QVQLVQSGAEVKKPGASVVKVSCKASGTYTFTSYGISMVRQAPGQGLEWMGWIS								
Consensus	(1)	QVQLVQSGAEVKKPGASVVKVSCKASGTYTFTSYGISMVRQAPGQGLEWMGWIS								
										Section 2
VH1-18	(53)	53	60	70	80	90				104
CR2-1.33_HC	(53)	AYNGNTNYAACKLQGRVTMTTDTSTSTAYMELRSLSRSDDTAVYYCAR-----								
Consensus	(53)	AYNGNTNYAACKLQGRVTMTTDTSTSTAYMELRSLSRSDDTAVYYCAR								
										Section 3
VH1-18	(105)	105	110	120						
CR2-1.33_HC	(99)	-----								
Consensus	(105)	SDYLYYYYGLDVWGQGTTVTVSS								
										SEQ ID NO:2
										SEQ ID NO:31

Figure 32B

										Section 1
										53
A20	(1)	1	10	20	30	40				
CR2-1.33_LC	(1)	DIQMTQSPSSLSASVGDRVTITCRASGIGSNYLWYQQKPGKVPKLLIYAST								
Consensus	(1)	DIQMTQSPSSLSASVGDRVTITCRASGIGSNYLWYQQKPGKVPKLLIYAST								
										Section 2
A20	(54)	54	60	70	80	90				106
CR2-1.33_LC	(54)	IQSGGVESRTSGBGSSTDFTLTISILOPEDVATYYCQKYN SAP-----								
Consensus	(54)	IQSGGVESRTSGBGSSTDFTLTISILOPEDVATYYCQKYN SAP-----								
										Section 3
A20	(107)	107	-							
CR2-1.33_LC	(107)	-----								
Consensus	(107)	-----								
										SEQ ID NO:9
										SEQ ID NO:32

SEQ ID NOS
 have been added

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FIGURE 33

Figure 33A

										Section 1
										51
(1) 1 10 20 30 40										
VH3-33 (1) QVQLVE8GGGVVQPGRSIRLSCAASGFTFSSYGMHIVVRQAPGKGLEWVAAI										
CR2-1.38.1_HC (1) QVQLVE8GGGVVQPGRSIRLSCAASGFTFSSYGMHIVVRQAPGKGLEWVAAI										
Consensus (1) QVQLVE8GGGVVQPGRSIRLSCAASGFTFSSYGMHIVVRQAPGKGLEWVAAI										
										Section 2
(52) 52 60 70 80 90 102										
VH3-33 (52) WYDG SNKRYADSVKGRFTISRDNSKNTLYILOMNSLRAEDTAVYYCAR ---										
CR2-1.38.1_HC (52) WYDG NDKYMADSVKGRFTISRDNSKNTLYILOMNSLRAEDTAVYYCAR										
Consensus (52) WYDG KYYADSVKGRFTISRDNSKNTLYILOMNSLRAEDTAVYYCAR										
										Section 3
(103) 103 110 127										
VH3-33 (99) -----										
CR2-1.38.1_HC (103) DSSDYLYYYYGMDVWGQGTTVTVSS										
Consensus (103)										

Figure 33B

										Section 1
										52
(1) 1 10 20 30 40										
A20 (1) DIQMTQSPSLSAISVGDRVTITCRASQGISNYIAWYQQKPGKVPKILLIYAA										
CR2-1.38.1_LC (1) DIQMTQSPSLSAISVGDRVTITCRASQGISNYIAWYQQKPGKVPNLLIYAA										
Consensus (1) DIQMTQSPSLSAISVGDRVTITCRASQGISNYIAWYQQKPGKVP LLIYAA										
										Section 2
(53) 53 60 70 80 90 104										
A20 (53) TIQSGVPSRSGSGSGTDFSLTISLQPEDVATY COKYNSAP ---										
CR2-1.38.1_LC (53) TIQSGVPSRSGSGSGTDFSLTISLQPEDVATY COKCNSAEWTFGOGTTV										
Consensus (53) TIQSGVPSRSGSGSGTDFSLTISLQPEDVATY COK NSAP										
										Section 3
(105) 105										
A20 (96) ---										
CR2-1.38.1_LC (105) BIR										
Consensus (105)										

SEQ ID NO:4
 SEQ ID NO:33

SEQ ID NO:9
 SEQ ID NO:34

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FIGURE 34

Figure 34A

	1	10	20	30	40	51	Section 1
VH5-51	(1) 1						
CR2-1.39.1_HC	(1) EVQLVQBGAEVKKPGESLKIISCKGSGYRFTSYWIGWVRQMPGKGLEWMGII						
Consensus	(1) EVQLVQSG EVKKPGESLKIISCKGSGY PTSYWIGWVRQMPGKGLEWMGII						
							Section 2
	52	60	70	80	90	102	
VH5-51	(52) YPGDSDTRYSPSPQGQVTISADRSISTAYLQWSSLKASDTAMYCAR						
CR2-1.39.1_HC	(52) YPGDSDTRYSPSPQGQVTISADRSISTAYLQWSSLKASDTAMYCAR						
Consensus	(52) YPGDSDTRYSPSPQGQVTISADRSISTAYLQWSSLKASDTAMYCAR						
							Section 3
	103	110	126				
VH5-51	(103) 99						
CR2-1.39.1_HC	(103) YYN8G8YYNVFDYWGQGTLTVSS						
Consensus	(103)						

Figure 34B

	1	10	20	30	40	52	Section 1
A30	(1) 1						
CR2-1.39.1_LC	(1) DIQMTQSPSISASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAA						
Consensus	(1) DIQMTQSPSISASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLIYAA						
							Section 2
	53	60	70	80	90	104	
A30	(53) SLOSGVPSRFSRSGSGSGTETLTISISSLQPEDRATTYCLOHNSYP						
CR2-1.39.1_LC	(53) SLOSGVPSRFSRSGSGSGTETLTISISSLQPEDRATTYCLOHNSYPWTFGQGTV						
Consensus	(53) SLOSGVPSRFSRSGSGSGTETLTISISSLQPEDRATTYCLOHNSYP						
							Section 3
	105	1087					
A30	(96) ---						
CR2-1.39.1_LC	(105) EZK						
Consensus	(105)						

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FIGURE 35

Figure 35A

	Section 1					
	1	10	20	30	40	52
VH1-8	(1) QVQLVQSGAEVKRPGASVVKV8CKASGYTFTSYDINWVRQATGQGLEWMGWNN					
CR2-1.45_HC	(1) QVQLVQSGAEVKRPGASVVKV8CKASGYTFTSYDINWVRQATGQGLEWMGWNN					
Consensus	(1) QVQLVQSGAEVKRPGASVVKV8CKASGYTFTSYDINWVRQATGQGLEWMGWNN					
	Section 2					
	53	60	70	80	90	104
VH1-8	(53) PNSGNTGYAQQKPFQGRVTMTRNTSISTAYMELSSRLSEDATAVYYCAR-----					
CR2-1.45_HC	(53) PNSGNTGYAQQKPFQGRVTMTRNTSISTAYMELSSRLSEDATAVYYCARGSGYSY					
Consensus	(53) PNSGNTGYAQQKPFQGRVTMTRNTSISTAYMELSSRLSEDATAVYYCAR					
	Section 3					
	105	110	125			
VH1-8	(99) -----					
CR2-1.45_HC	(105) GYDYYYGMDVWGQGTTVTVS					
Consensus	(105)					

SEQ ID NO:1
SEQ ID NO:38

Figure 35B

	Section 1					
	1	10	20	30	40	53
A20	(1) D1QMTQSPSSLSASVGDRVTITCRASQG1SNYLAWYQQKPGKVKPLLIYAAST					
CR2-1.45_LC	(1) D1QMTQSPSSLSASVGDRVTITCRASQG1SNYLAWYQQKPGKVKPLLIYAAST					
Consensus	(1) D1QMTQSPSSLSASVGDRVTI CRASQG1SN LAWYQQKPGKVKPLLIYAAST					
	Section 2					
	54	60	70	80	90	106
A20	(54) D1QGVEFRESGSGSGTDFLTISLQPEDAVATYYCQKYN5AP-----					
CR2-1.45_LC	(54) D1QGVEFRESGSGSGTDFLTISLQPEDAVATYYCQKYN5APFTPGPGTKVDI					
Consensus	(54) D1Q GVEFRESGSGSGTDFLTISLQPEDAVATYYCQKYN5AP					
	Section 3					
	107	110				
A20	(96) -----					
CR2-1.45_LC	(107) K					
Consensus	(107)					

SEQ ID NO:9
SEQ ID NO:39

SEQ ID NOS
have been added

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FIGURE 36

Figure 36A

	1	10	20	30	40	51	Section 1
VH1-8	(1) QVQLVQSGAEVKPGASVKVSKRASGY	FTSYDINWVRQATGQGLEWMGWM					
CR2-1.46.1_HC	(1) QVQLVQSGAEVKPGASVKVSKRASGY	FTSYDINWVRQATGQGLEWMGWM					
Consensus	(1) QVQLVQSGAEVKPGASVKVSKRASGY	FTSYDINWVRQATGQGLEWMGWM					
	52	60	70	80	90	102	Section 2
VH1-8	(52) NPNNSGNTGYAQKFQGRVITMTRNTSISTAYMELSSLRSEMTAVYYCAR	---					
CR2-1.46.1_HC	(52) NPNNGNTGYAQKFQGRVITMTRNTSISTAYMELSSLRSEMTAVYYCAR	IVV					
Consensus	(52) NPN GNTGYAQKFQGRVITMTRNTSISTAYMELSSLRSEMTAVYYCAR	IVV					
	103	110	126				Section 3
VH1-8	(103) (99) -----			SEQ ID NO:1			
CR2-1.46.1_HC	(103) VVTATDYYGMDVWGQGTTVSS			SEQ ID NO:40			
Consensus	(103)						

Figure 36B

	1	10	20	30	40	52	Section 1
A30	(1) DIQMTQSPSISASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLLIAAS						
CR2-1.46.1_LC	(1) DIQMTQSPSISASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLLIAAS						
Consensus	(1) DIQMTQSPSISASVGDRVTITCRASQGIRNDLGWYQQKPGKAPKRLLIAAS						
	53	60	70	80	90	104	Section 2
A30	(53) SIQSGVPSRFSGSGTEFTLTISBLOPEDFATTYCLOHNSYP	-----					
CR2-1.46.1_LC	(53) SIQSGVPSRFSGSGTEFTLTISBLOPEDFATTYCLOHSGYPPTFGQGTV						
Consensus	(53) SIQSGVPSRFSGSGSGTEFTLTISBLOPEDFATTYCLOHSGYPPTFGQGTV						
	105	1067					Section 3
A30	(105) (96) -----	SEQ ID NO:11					
CR2-1.46.1_LC	(105) EIK	SEQ ID NO:41					
Consensus	(105)						

SEQ ID NOS
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FIGURE 37

Figure 37A

	(1)	10	20	30	40	51	Section 1
CR2-1.48.1_HC	(1) QVQLVQSGAEVVKPGASVKVSKRASGTYFTSYGIISWVROAPGOGLEWMGWI						
VH1-18	(1) QVQLVQSGAEVVKPGASVKVSKRASGTYFTSYGIISWVROAPGOGLEWMGWI						
Consensus	(1) QVQLVQSGAEVVKPGASVKVSKRASGTYFTSYGIISWVROAPGOGLEWMGWI						
	(52)	60	70	80	90	102	Section 2
CR2-1.48.1_HC	(52) SAYNGNTNYAQKIQGRVTMTTDTSITSTAYMELREIRRSDDITAVYYCARVEY						
VH1-18	(52) SAYNGNTNYAQKIQGRVTMTTDTSITSTAYMELRSLRSDITAVYYCAR---						
Consensus	(52) SAYNGNTNYAQKIQGRVTMTTDTSITSTAYMELRSLRSDITAVYYCAR						
	(103)	103	110	125			Section 3
CR2-1.48.1_HC	(103) YYDGSGYYYFDYWGQGTIVTVSS						
VH1-18	(99) -----						
Consensus	(103)						

Figure 37B

	(1)	10	20	30	40	52	Section 1
CR2-1.48.1_LC	(1) DIQMTQSPSISVSVASVGDRVTITCRASQGIGSSWLAWSQQKPGKAPKLLIYAA						
L5	(1) DIQMTQSPSISVSVASVGDRVTITCRASQGIGSSWLAWSQQKPGKAPKLLIYAA						
Consensus	(1) DIQMTQSPSISVSVASVGDRVTITCRASQGIGSSWLAWSQQKPGKAPKLLIYAA						
	(53)	60	70	80	90	104	Section 2
CR2-1.48.1_LC	(53) ILQSGVPSRFSGSGSGTDFTLTISIQLQPEDFAIYYCQQINSFPRTFGQGTV						
L5	(53) SLQSGVPSRFSGSGSGTDFTLTISIQLQPEDFAIYYCQQINSFP-----						
Consensus	(53) ILQSGVPSRFSGSGSGTDFTLTISIQLQPEDFAIYYCQQANSFP						
	(105)	105					Section 3
CR2-1.48.1_LC	(105) EIK SEQ ID NO:43						
L5	(96) --- SEQ ID NO:7						
Consensus	(105)						

SEQ ID NOS
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FIGURE 38

Figure 38A

	1	10	20	30	40	51	Section 1
CR2-1.49.1_HC	(1)	QVQLVQSGAEVKKPGASVVKVSKRASGYTFTSYDINWVRQATGQGLEWMGWM					
VH1-8	(1)	QVQLVQSGAEVVKVSKRASGYTFTSYDINWVRQATGQGLEWMGWM					
Consensus	(1)	QVQLVQSGAEVKKPGASVVKVSKRASGYTFTSYDINWVRQATGQGLEWMGWM					Section 2
	(52)	52	60	70	80	90	102
CR2-1.49.1_HC	(52)	NPNSGDTGYAQQKFQGRVTMTRNTSISTAYMELSSLRSEDTAVYFCAR	MRDI				
VH1-8	(52)	NPNSGNTGYAQQKFQGRVTMTRNTSISTAYMELSSLRSEDTAVYFCAR	---				
Consensus	(52)	NPNSG TGYAQQKFQGRVTMTRNTSISTAYMELSSLRSEDTAVYFCAR					Section 3
	(103)	103	110	127			
CR2-1.49.1_HC	(103)	VATYYYYFYGMDVWGQGTTVTVSS					
VH1-8	(99)	-----					
Consensus	(103)						

SEQ ID NO:44
 SEQ ID NO:1

Figure 38B

	1	10	20	30	40	52	Section 1
CR2-1.49.1_LC	(1)	DIVMTQSPLSLPVTPGEPAISCRSBSQSLHSNGYNYLDWYILKPGQSPOLL					
A19	(1)	DIVMTQSPLSLPVTPGEPAISCRSBSQSLHSNGYNYLDWYILKPGQSPOLL					
Consensus	(1)	DIVMTQSPLSLPVTPGEPAISCRSBSQSLHSNGYNYLDWYILKPGQSPOLL					Section 2
	(53)	53	60	70	80	90	104
CR2-1.49.1_LC	(53)	IYLGSRASGVPDFRSGSGSGTDFTLKISRVEAEDVGVYYCMOTIOTITFGQ					
A19	(53)	IYLGSRASGVPDFRSGSGSGTDFTLKISRVEAEDVGVYYCMQALQTP	---				
Consensus	(53)	IYLGSRASGVPDFRSGSGSGTDFTLKISRVEAEDVGVYYCMQALQTP					Section 3
	(105)	105	111				
CR2-1.49.1_LC	(105)	GTRLEIR					
A19	(101)	-----					
Consensus	(105)						

SEQ ID NO:45
 SEQ ID NO:8

SEQ ID NOS
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FIGURE 39

Figure 39A

	(1)	10	20	30	40	51	Section 1
CR2-1.51.1_HC	(1)	EVQLVQSGAEVKKPGESLKISCKGSGYSPFTSYWIGWVRQMPGKGLEWMGII					
VH5-51	(1)	EVQLVQSGAEVKKPGESLKISCKGSGYSPFTSYWIGWVRQMPGKGLEWMGII					
Consensus	(1)	EVQLVQSGAEVKKPGESLKISCKGSGYSPFTSYWIGWVRQMPGKGLEWMGII					
	(52)	60	70	80	90	102	Section 2
CR2-1.51.1_HC	(52)	YEGDSDA	YPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYCARHYDY				
VH5-51	(52)	YFGDSDT	YPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYCAR				
Consensus	(52)	YEGDSD	YPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYCAR				
	(103)	103	110	126			Section 3
CR2-1.51.1_HC	(103)	WWRNYRYTGWFDPWGQGTLTVSS					
VH5-51	(99)	-----					
Consensus	(103)	-----					

SEQ ID NO:46
 SEQ ID NO:6

Figure 39B

	(1)	10	20	30	40	52	Section 1
CR2-1.51.1_LC	(1)	EIVITQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKEGQAPRILLYGA					
A27	(1)	EIVITQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKEGQAPRILLYGA					
Consensus	(1)	EIVITQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKEGQAPRILLYGA					
	(53)	60	70	80	90	104	Section 2
CR2-1.51.1_LC	(53)	SNRNAGCIEDRPSGSGSGTDTISRLPEDPAVYQQYGSLLPTFPGBTK					
A27	(53)	SNRNAGCIEDRPSGSGSGTDTISRLPEDPAVYQQYGSLLPTFPGBTK					
Consensus	(53)	S RATGIPDRPSGSGSGTDTISRLPEDPAVYQQYGSLLPTFPGBTK					
	(105)	10508					Section 3
CR2-1.51.1_LC	(105)	VDIK	SEQ ID NO:47				
A27	(97)	-----	SEQ ID NO:10				
Consensus	(105)	-----	-----				

SEQ ID NO:
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FIGURE 40

Figure 40A

	1	10	20	30	40	52	Section 1
Cur2-6.4.1 hc	(1)						
VH1-8	(1)	QVOLVQSGAEVKKPGASVVKVSCRA	GYTFTSYDINWV	VRQATGQGL	EWMGWIN		
Consensus	(1)	QVOLVQSGAEVKKPGASVVKVSCRA	GYTFTSYDINWV	VRQATGQGL	EWMGWIN		
	53	60	70	80	90	104	Section 2
Cur2-6.4.1 hc	(53)	PN	SGNTD	ACKF	QGRV	TMTRD	TSISTAYMELSS
VH1-8	(53)	PN	SGNTGY	ACKF	QGRV	TMTRNTS	TSISTAYMELSS
Consensus	(53)	PN	SGNT	YACKF	QGRV	TMTR	TSISTAYMELSS
	105	110	125				Section 3
Cur2-6.4.1 hc	(105)	NYDYYYGMDVWGQGTTV	V83				SEQ ID NO:48
VH1-8	(99)	-----	-----				SEQ ID NO:1
Consensus	(105)	-----	-----				

Figure 40B

	1	10	20	30	40	52	Section 1
Cur2-6.4.1 Lc	(1)						
A27	(1)	EIVLTQSGPTL	SLSPGERATL	SCRASQSVSSSYL	AWYQQKPGQAPRLLIYA		
Consensus	(1)	EIVLTQSGPTL	SLSPGERATL	SCRASQSVSSSYL	AWYQQKPGQAPRLLIYA		
	53	60	70	80	90	104	Section 2
Cur2-6.4.1 Lc	(53)	SSRATGIPDRF	SGSGSGT	DFTLT	ISRL	PEPEDFAVYYCQQYGS	SPCSFGQG
A27	(53)	SSRATGIPDRF	SGSGSGT	DFTLT	ISRL	PEPEDFAVYYCQQYGS	SP
Consensus	(53)	SSRATGIPDRF	SGSGSGT	DFTLT	ISRL	PEPEDFAVYYCQQYGS	SP
	105	105	08				Section 3
Cur2-6.4.1 Lc	(105)	LEIR					
A27	(97)	-----					
Consensus	(105)	-----					

Seq ID NOS
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A Fully Human Monoclonal Antibody (CR002) Identifies PDGF-D as a Novel Mediator of Mesangioproliferative Glomerulonephritis

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Abstract. PDGF-B is of central importance in mesangioproliferative diseases. PDGF-D, a new PDGF isoform, like PDGF-B, signals through the PDGF $\beta\beta$ -receptor. The present study first determined that PDGF-D is mitogenic for rat mesangial cells and is not inhibited by a PDGF-B antagonist. Low levels of PDGF-D mRNA were detected in normal rat glomeruli. After induction of mesangioproliferative nephritis in rats by anti-Thy 1.1 mAb, glomerular PDGF-D mRNA and protein expression increased significantly from days 4 to 9 in comparison with nonnephritic rats. Peak expression of PDGF-D mRNA occurred 2 d later than peak PDGF-B mRNA expression. In addition, PDGF-D serum levels increased significantly in the nephritic animals on day 7. For investigating the functional role of PDGF-D, neutralizing fully human mAb were generated using the XenoMouse technology. Rats with anti-Thy 1.1-induced nephritis were treated on days 3 and 5 with different amounts of a fully human PDGF-DD-specific neutralizing mAb (CR002), equal amounts of irrelevant

control mAb, or PBS by intraperitoneal injection. Specific antagonism of PDGF-D led to a dose-dependent (up to 67%) reduction of glomerular cell proliferation. As judged by double immunostaining for 5-bromo-2'-deoxyuridine and α -smooth muscle actin, glomerular mesangial cell proliferation was reduced by up to 57%. Reduction of glomerular cell proliferation in the rats that received CR002 was not associated with reduced glomerular expression of PDGF-B mRNA. PDGF-D antagonism also led to reduced glomerular infiltration of monocytes/macrophages (day 5) and reduced accumulation of fibronectin (day 8). In contrast, no effect was noted in normal rats that received an injection of CR002. These data show that PDGF-D is overexpressed in mesangioproliferative states and can act as an auto-, para-, or even endocrine glomerular cell mitogen, indicating that antagonism of PDGF-D may represent a novel therapeutic approach to mesangioproliferative glomerulonephritides.

For two decades, the PDGF system consisted of two PDGF chains, PDGF-A and -B, that are secreted as homo- or heterodimers and bind to dimeric PDGF receptors composed of α - and/or β -chains. Whereas PDGF-A binds to the α -chain only, PDGF-B is a ligand for all receptor types (1). Recently, two novel PDGF isoforms, designated PDGF-C and -D, that are released as homodimers, PDGF-CC and -DD, were described (2–4). The core chain of PDGF-CC seems to be largely a ligand for the PDGF $\alpha\alpha$ -receptor, whereas PDGF-DD binds predominantly to the PDGF $\beta\beta$ -receptor (2–4). In both cases,

some binding has also been described to the $\alpha\beta$ -receptor (2,4,5). All four PDGF isoforms, as well as both receptor chains, are expressed in the kidney, albeit in distinct spatial arrangements (1,6,7).

Many progressive renal diseases, including diabetic nephropathy, as well as the most frequent types of glomerulonephritides, such as IgA nephropathy, are characterized by glomerular mesangial cell proliferation and/or matrix accumulation (8). Ample evidence is now available to link the PDGF system, in particular PDGF-B-chain, to both of these processes given that (1) mesangial cells produce PDGF-B *in vitro* and various growth factors induce mesangial proliferation via induction of auto- or paracrine PDGF-B-chain excretion (9–12); (2) PDGF-B-chain and its receptor are overexpressed in many glomerular diseases (13–17); (3) infusion of PDGF-BB or glomerular transfection with a PDGF-B-chain cDNA can induce selective mesangial cell proliferation and matrix accumulation *in vivo* (18,19); (4) PDGF-B-chain or β -receptor knockout mice fail to develop a mesangium (20,21); and (5) specific inhibition of PDGF-B using antibodies, aptamers, soluble PDGF receptors, or PDGF β -receptor tyrosine kinase blockers not only results in a diminution of mesangiopro-

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liferative changes (reviewed in (1) and (22–24)) but, more important, was also able to prevent long-term renal scarring (25). Apart from its role in glomerular pathology, PDGF-B-chain may also contribute to renal interstitial fibrosis, *i.e.*, the common final pathway of almost all progressive renal diseases. Thus, we have reported that both PDGF β -receptor subunit and PDGF-B-chain are overexpressed in renal interstitial fibrosis (26), and Tang *et al.* (27) demonstrated that infusion of large doses of PDGF-BB alone is able to induce interstitial fibrotic changes in normal rat kidney.

Given that PDGF-D, like the PDGF-B-chain, can bind to the $\beta\beta$ - and $\alpha\beta$ -receptor, their biologic activities in the kidney may be similar, albeit not identical, in view of the differential binding to the PDGF $\alpha\alpha$ -receptor. PDGF-DD is secreted as a disulphide-linked homodimer, which is activated upon limited proteolysis with dissociation of its CUB-domain to become a specific agonistic ligand for PDGF $\beta\beta$ - and $\alpha\beta$ -receptor (2,4). In developing and adult normal kidney, PDGF-D is expressed in visceral glomerular epithelial cells and some vascular smooth muscle cells (6). In the developing mouse kidney, only cells of the branching ureter exhibited PDGF-D immunoreactivity (4). No other information on PDGF-D and the kidney is currently available. In the present study, we investigated whether PDGF-D mediated glomerular mesangial cell proliferation and matrix accumulation *in vitro* and *in vivo*.

Materials and Methods

Reagents

Recombinant human and murine PDGF-DD lacking the CUB-domain, *i.e.* biologically active PDGF-DD p35, was produced as described (2). Human PDGF-CC was produced by the same protocol. PDGF-AA and PDGF-BB were purchased from R & D Systems (Minneapolis, MN). Immunohistochemical analysis was performed with a PDGF-DD-specific rabbit polyclonal antibody as described previously (15).

Fully human PDGF-DD mAb were generated as described previously (28) with the following modifications. Briefly, the human IgG2-bearing XenoMouse strain (8 to 10 wk old) was immunized twice weekly by footpad injection with 10 μ g of V5-tagged soluble PDGF-DD (2) in complete Freund's adjuvant (28). Hybridomas were generated using electrocell fusion. Fully human isotype matched mAb PK16.3 was used as the negative control.

Characterization of the Neutralizing Anti-PDGF-D mAb CR002.6.4

PDGF solid-phase ELISA was performed by coating Corning 96-well flat-bottom high-protein binding polystyrene microtiter plates with 500 ng/ml human PDGF-AA, PDGF-BB, PDGF-CC, or PDGF-DD or murine PDGF-DD overnight. Plates were blocked with Assay Diluent (Pharmingen, San Diego, CA) for 1 h. PDGF-DD mAb CR002.6.4 or control mAb PK16.3 was then added at the indicated concentration for 2 h. Primary mAb binding was detected using anti-human horseradish peroxidase-conjugated secondary antibody with TMB Reagent (Pharmingen). Microtiter plates were read at 450 nm with a Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA).

For Western blot analysis, human PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD (250 ng) or glomerular protein lysates (20 μ g) isolated from rats with anti-Thy 1.1 nephritis day 8 after disease induction were diluted in SDS-PAGE sample buffer, boiled, and

subjected to 16% SDS-PAGE (human PDGF) or 4 to 20% SDS-PAGE (rat glomerular protein lysates) under nonreducing conditions. Proteins were transferred to Hybond-P membranes (Amersham), and filters were probed with mAb CR002.6.4 or control mAb PK16.3 (0.85 μ g/ml) for 12 h. After washing, filters were incubated with anti-human horseradish peroxidase-conjugated secondary antibody. Bands were visualized by enhanced chemiluminescence (Amersham).

The NIH 3T3 and normal human lung fibroblast neutralization assay was performed as described previously (2) with the following modifications. Briefly, cells were serum-starved 24 or 18 h, respectively, and mAb were added at the indicated concentration. Human or murine PDGF-DD was then added at 100 ng/ml. After 18 h, 5-bromo-2'-deoxyuridine (BrdU) was added for 5 h and incorporation-assayed according to the manufacturer's specifications (Roche).

Aptamer-Based Antagonist against PDGF

The synthesis and characterization of the PDGF-B aptamer (NX1975) have been described in detail (29). Modifications of the original DNA aptamer involved substitutions of certain nucleotides with 2-fluoropyrimidines and 2'-O-methylpurines to improve nuclease resistance as well as coupling of the aptamer to 40-kD polyethylene glycol to prolong its plasma residence time *in vivo* (22).

Mesangial Cell Culture Experiments

Rat mesangial cells were established in culture, characterized, and maintained as described previously (30). For examining the proliferative effect of PDGF-DD, rat mesangial cells were seeded in 96-well plates (Nalge Nunc, Naperville, IL), grown to subconfluence, and growth-arrested for 48 h in RPMI 1640 with 1% BSA. After 48 h, human PDGF-DD (10 to 200 ng/ml) and human PDGF-BB (10 and 50 ng/ml) together with PDGF-B-chain aptamer (100 ng/ml) or sequence-scrambled aptamer (100 ng/ml) were added and the cells were incubated for 24 h. DNA synthesis was determined by a BrdU incorporation assay according to the manufacturer's instructions (Roche).

Glomerular RNA Extraction and Analyses

Total RNA was extracted from isolated rat glomeruli and adrenal gland with the guanidinium isothiocyanate/phenol/chloroform method using standard procedures (31). The RNA content and sample purity were determined by UV spectrophotometry at 260 and 280 nm. The cDNA syntheses were performed in a 30- μ l reaction mix including 1 μ g of total RNA, 1 μ l of random primer (6 nt, 250 ng/ μ l; Roche), 6 μ l of M-MLV reverse transcriptase buffer (Invitrogen, Carlsbad, CA), 1.5 μ l of dNTP-mix (10 mM each, Amersham Pharmacia Biotech), 0.7 μ l of RNase-inhibitor (40 U/ μ l, Promega), 1 μ l of M-MLV reverse transcriptase (200 U/ μ l; Invitrogen), and DEPC-treated H₂O. The mix was incubated for 10 min at 25°C followed by 1 h at 42°C.

Real-time quantitative PCR was carried out using an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). In each reaction, 0.75 μ l of cDNA and 12.5 μ l of PCR Master Mix (Platinum Quantitative PCR SuperMix-UDG with ROX Reference Dye; Invitrogen) were used in a total of 25- μ l volume. The PCR conditions were 50°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Taqman primers and probes were designed from sequences in the GenBank database using the Primer Express software (Applied Biosystems). The sequences of primers and probes used in this study are listed in Table 1.

Rat Model of Mesangioproliferative Glomerulonephritis

All animal experiments were approved by the local review boards. Anti-Thy 1.1 mesangial proliferative glomerulonephritis was induced

Table 1. Primers and probes

Gene	Forward Primer	Reverse Primer	Taqman Probe
Rat GAPDH	5'-ACAAGATGGTAAGGTCGGT-3'	5'-AGAAGGCAGCCCTGGTAACC-3'	5'-CGGATTGGCCGTATCGGACGC-3'
Rat PDGF-A	5'-TTCTTGATCTGGCCCCAT-3'	5'-TTGACGCTGCTGGTGTACAG-3'	5'-CAGTCAGCGCTCACCTCCACA-3'
Rat PDGF-B	5'-GCAAGACCGCTACAGAGGTG-3'	5'-GAAGTTGGCATTGGTGCAG-3'	5'-TCCAGATCTCGCGGAACCTCATCG-3'
Rat PDGF-C	5'-CAGCAAGTTGCAGCTCTCCA-3'	5'-GACAACCTCTCATGCCGGG-3'	5'-CGACAAGGAGCAGAACGGAGTCAA-3'
Rat PDGF-D	5'-ATCGGGACACTTTGCGACT-3'	5'-GTGCCTGTCACCCGATGTT-3'	5'-TTGCGCAATGCCAACCTCAGGAG-3'

in male Wistar rats (Charles River) weighing 180 g by injection of 1 mg/kg anti-Thy 1.1 mAb (clone OX-7; European Collection of Animal Cell Cultures, Salisbury, England).

For studying the kinetics of PDGF-D expression during anti-Thy 1.1 nephritis, 45 rats received anti-Thy 1.1 mAb and were killed at time points 4 h and days 1, 2, 4, 7, 9, 14, 21, and 28 after mAb injection ($n = 5$ each). After the animals were killed, renal tissue and isolated glomeruli were obtained. Glomerular isolation was performed by differential sieving (32). Glomerular isolates were checked microscopically and exhibited a purity of >95%. In addition, adrenal tissue was obtained.

For studying the effects of PDGF-DD antagonism *in vivo*, rats were treated with the anti-PDGF-D mAb CR002.6.4, control mAb PK16.3, or PBS on days 3 and 5 after disease induction. Treatment consisted of intraperitoneal injections of the mAb dissolved in 800 μ l of 20 mM Tris-HCl/100 mM NaCl (pH 7.4). mAb timing was chosen to treat rats from approximately 1 d after onset to the peak of mesangial cell proliferation, which in the OX-7-induced anti-Thy 1.1 nephritis model occurs between days 5 and 8 after disease induction. We investigated the *in vivo* effects of three different doses of the anti-PDGF-D mAb. The average dose of 10 mg (day 3) plus 4 mg (day 5) anti-PDGF-D mAb CR002.6.4/kg body wt was chosen on the basis of calculations that this would result in serum levels of >50 μ g/ml. For verifying that relevant levels of CR002.6.4 or irrelevant control IgG2 PK16.3 were achieved, human IgG2 serum levels were measured in treatment groups 1 to 4 (see below) on days 5 and 8. Animals with levels <30 μ g/ml on day 5 were excluded from the analyses. Altogether, seven groups of rats with sufficient human serum IgG2 in the mAb-treated groups were studied:

1. Seven nephritic rats that received 5 mg/kg body wt CR002.6.4 on day 3 and 2 mg/kg on day 5
2. Seven nephritic rats that received 10 mg/kg body wt CR002.6.4 on day 3 and 4 mg/kg on day 5
3. Eight nephritic rats that received 20 mg/kg body wt CR002.6.4 on day 3 and 8 mg/kg on day 5
4. Eight nephritic rats that received 20 mg/kg body wt irrelevant control PK16.3 on day 3 and 8 mg/kg on day 5
5. Nine nephritic rats that received equivalent injections of PBS alone
6. Five nonnephritic, normal rats that received 10 mg/kg body wt of CR002.6.4 on day 3 and 4 mg/kg on day 5 and
7. Five non-nephritic, normal rats that received 10 mg/kg body wt irrelevant control PK16.3 on day 3 and 4 mg/kg on day 5

In four randomly selected rats each from groups 1 to 5, renal biopsies for histologic evaluation were obtained on day 5 by intravital biopsy as described (22). In all rats, post mortem biopsy was obtained on day 8 after disease induction. The remaining cortical tissue of two or three rats from every group was then pooled and used to isolate glomeruli (see above). Urine collections were performed on day 7 after disease in-

duction. BrdU (100 mg/kg body wt; Sigma, St. Louis, MO) was injected intraperitoneally 4 h before killing on day 8.

Renal Morphology

Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy's solution and embedded in paraffin. Four-microgram sections were stained with the periodic acid-Schiff reagent and counterstained with hematoxylin. In the periodic acid-Schiff-stained sections, the number of mitoses in >30 cross-sections (range, 30 to 100) of consecutive cortical glomeruli containing >20 discrete capillary segments each was evaluated by one of the authors, who was unaware of the origin of the slides. Mesangiolysis was graded as described (33) on a semiquantitative scale: 0 = no mesangiolysis, 1 = segmental mesangiolysis, 2 = global mesangiolysis, 3 = microaneurysm.

Immunoperoxidase Staining

Four-microgram sections of methyl Carnoy's fixed biopsy tissue were processed by an indirect immunoperoxidase technique (34). Primary antibodies were identical to those described previously (33,35) and included a murine mAb (clone 1A4) to α -smooth muscle actin; a murine mAb (clone PGF-007) to PDGF-B-chain; a murine mAb (clone ED1) to a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells; a polyclonal goat antibody to human type I collagen (Southern Biotechnology Associates, Birmingham, AL); an affinity-purified IgG fraction of a polyclonal rabbit anti-rat fibronectin antibody (Chemicon, Temecula, CA); plus appropriate negative controls as described previously (33,35). PDGF-DD was detected by a polyclonal rabbit antibody to human PDGF-DD. Serum was purified by Protein A Sepharose chromatography. PDGF-CC cross-reactivity was eliminated by absorption to a PDGF-CC affinity column. The resulting Ig flowthrough was concentrated and did not react with PDGF-AA, -BB, or -CC by ELISA or Western blot analysis.

For obtaining mean numbers of infiltrating leukocytes in glomeruli, >50 consecutive cross-sections of glomeruli were evaluated and mean values per kidney were calculated. For the evaluation of the immunoperoxidase stains for type I collagen, fibronectin, and α -smooth muscle actin, each glomerular area was graded semiquantitatively and the mean score per biopsy was calculated. Each score reflects mainly changes in the extent rather than the intensity of staining and depends on the percentage of the glomerular tuft area showing focally enhanced positive staining: 1 = 0 to 25%, 2 = 25 to 50%, 3 = 50 to 75%, 4 = >75% (22). Evaluation of all slides was performed by an observer, who was unaware of the origin of the slides.

Immunohistochemical Double Staining

Double immunostaining to identify the type of proliferating cells was performed as reported previously (26) by first staining sections for proliferating cells with a murine mAb (clone BU-1) against

BrdU-containing nuclease in Tris-buffered saline (Amersham) using an indirect immunoperoxidase procedure. Sections were then incubated with the IgG₁ mAb 1A4 raised against α -smooth muscle actin. Cells were identified as proliferating mesangial cells when they showed positive nuclear staining for BrdU and when the nucleus was completely surrounded by cytoplasm positive for α -smooth muscle actin. Negative controls included omission of either of the primary antibodies, in which case no double staining was noted.

Serum Measurements of PDGF-DD and Anti-CR002.6.4 mAb

Circulating levels of rat PDGF-DD were measured using a PDGF-DD-specific sandwich ELISA as described previously with modifications to allow accurate quantification of rodent forms of PDGF-DD (36). The two fully human mAb (CR002.1.6 and CR002.1.17) used in the sandwich ELISA recognized different epitopes on the PDGF-DD molecule (data not shown). The ELISA was performed as follows: 50 μ l of capture mAb (CR002.1.6) in coating buffer (0.1 M NaHCO₃, pH 9.6) at a concentration of 2 μ g/ml was coated on ELISA plates (Fisher). After incubation at 4°C overnight, the plates were treated with 200 μ l of blocking buffer (0.5% BSA, 0.1% Tween 20, 0.01% Thimerosal in PBS) for 1 h at 25°C. The plates were washed (3×) with washing buffer (WB; 0.05% Tween 20 in PBS) and were incubated with serum samples overnight at 4°C, washed with WB, and then incubated with 100 μ l/well biotinylated detection mAb CR002.1.17 for 1 h at 25°C. After washing, the plates were incubated with horseradish peroxidase-streptavidin for 15 min, washed as before, and then treated with 100 μ l/well o-phenylenediamine in H₂O₂ (Sigma developing solution) for color generation. The reaction was stopped with 2 M H₂SO₄ and analyzed using an ELISA plate reader at 492 nm. Concentration of rat PDGF-DD in serum samples was calculated by comparison with dilutions of purified CR002.6.4 mAb using a four-parameter curve-fitting program.

Circulating levels of anti-PDGF-DD mAb were measured using a sandwich ELISA specific for human IgG as modified from the protocol previously described. Quantification of the fully human anti-

PDGF-D mAb CR002.6.4 used an ELISA with 96-well plates (Fisher) coated with 100 μ l/well goat anti-human Fc-specific IgG (CalTag) at 2 μ g/ml in coating buffer. After incubation at 4°C overnight, the plates were washed (3×) using WB, blocked with 200 μ l of blocking buffer in PBS for 2 h at 25°C, then washed three times in WB. Selected wells of the ELISA plate were incubated with 50 μ l/well human κ Ig standard in duplicate diluted to seven threefold serial dilutions from 1000 ng/ml in blocking buffer, or 50 μ l/well of samples serially diluted in blocking buffer. Plates were allowed to incubate at room temperature for 2 h and then washed three times with WB. Goat anti-human κ -horseradish peroxidase (1:2000 in blocking buffer) was added to the plates at 100 μ l/well, incubated at room temperature for 1 h, then washed three times with WB. ELISA plates were treated with 100 μ l/well o-phenylenediamine in H₂O₂ (Sigma developing solution) for color development. The reaction was stopped with 50 μ l/well H₂SO₄ (2 M) and analyzed using an ELISA plate reader at 492 nm. Concentration of anti-PDGF-D mAb in serum samples was calculated by comparison with dilutions of purified CR002.6.4 mAb using a four-parameter curve-fitting program.

Miscellaneous Measurements

Urinary albumin levels were determined with an ELISA kit specific for rat albumin (Nephrat, Exocell). Urinary creatinine was determined by the method of two-point kinetics with a Vitros 250 analyzer (Orthoclinical Diagnostics). All measurements were performed in duplicate. BP measurements were performed by the tail-cuff method, using a programmed sphygmomanometer, BP-98A (Softron) (37). Measurements of total hemolytic activity of the classic complement pathway (CH₅₀) and hemolytic activity of the alternative complement pathway (AP₅₀) were performed in sera of rats using appropriate assays (Total Hemolytic Complement and Alternative Pathway Hemolytic Complement kits; The Binding Site, Birmingham, England) according to the manufacturer's instruction. All measurements were performed in duplicate.

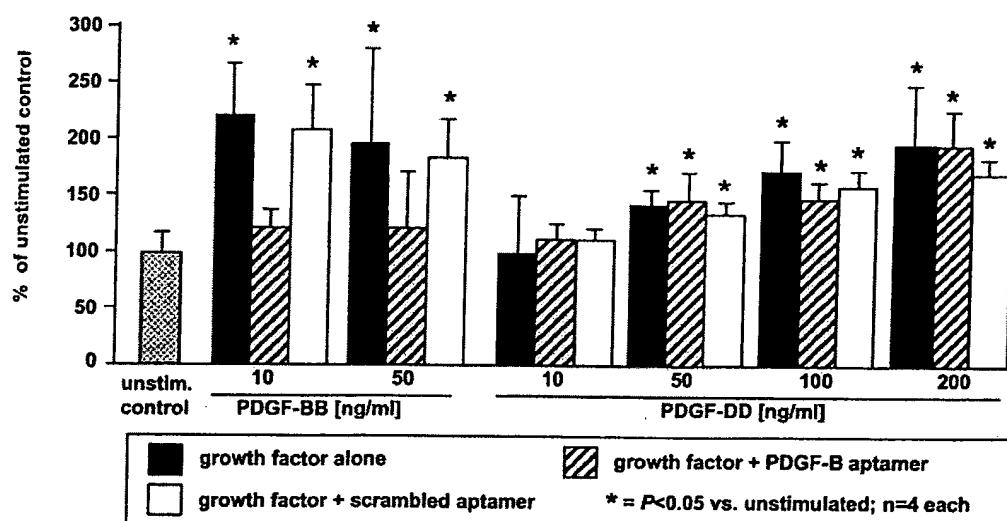


Figure 1. PDGF-DD acts as a growth factor for mesangial cells *in vitro*. 5-Bromo-2'-deoxyuridine (BrdU) incorporation in growth-arrested rat mesangial cells stimulated with human PDGF-BB (10 and 50 ng/ml) and human PDGF-DD (10 to 200 ng/ml) with or without PDGF-B aptamer (100 ng/ml) or sequence scrambled aptamer control (100 ng/ml). Data are means \pm SD of four independent experiments. * $P < 0.05$ versus unstimulated control.

Statistical Analyses

All values are expressed as means \pm SD. Statistical significance (defined as $P < 0.05$) was evaluated using ANOVA and Bonferroni t tests.

Results

PDGF-DD Is a Mitogen for Mesangial Cells In Vitro

Incubation of growth-arrested cultured rat mesangial cells with PDGF-DD led to a dose-dependent increase in proliferation (Figure 1). Independence of the mitogenic PDGF-DD activity from PDGF-BB was demonstrated by incubating the cells with antagonistic PDGF-BB aptamers or sequence-scrambled control aptamers simultaneously to PDGF-DD. Whereas the aptamers blocked PDGF-BB-induced proliferation, they had no effect on the mitogenic potential of PDGF-DD (Figure 1). Similar data were obtained with human mesangial cells (not shown).

PDGF-DD Is Overexpressed in Glomeruli during Mesangiproliferative Nephritis

After the induction of mesangiproliferative anti-Thy 1.1 nephritis in rats, glomerular PDGF-D mRNA expression initially decreased 0.4-fold at 4 h after disease induction but then increased to 2.4- and 2.9-fold at days 7 and 9, respectively, in comparison with nonnephritic rats (Figure 2). This latter peak paralleled that of glomerular PDGF-A mRNA expression and occurred with some delay after the maximum PDGF-B mRNA expression (Figure 2). In contrast to these three PDGF isoforms, PDGF-C mRNA was not upregulated during the first 28 d of anti-Thy 1.1 nephritis. To assess whether PDGF-D mRNA upregulation during anti-Thy 1.1 nephritis is specific for the kidney, we also investigated adrenal mRNA levels, because the adrenal gland has been noted to be a prominent source of PDGF-D (2). In contrast to glomeruli, no significant

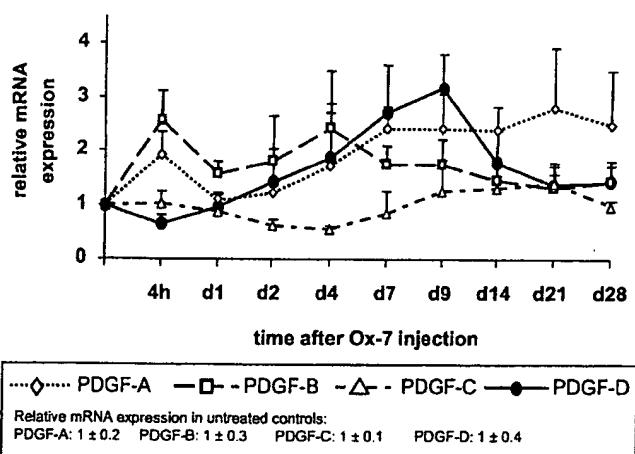


Figure 2. Transcript expression of PDGF-A, -B, -C, and -D in the course of anti-Thy 1.1 nephritis. Rats were killed at 4 h and at days 1, 2, 4, 7, 9, 14, 21, and 28 after disease induction ($n = 5$ each). RNA was isolated from the glomeruli, and the expression was measured by real-time quantitative PCR. The figure shows the transcript expression relative to the expression in untreated rats.

change in the PDGF-D mRNA expression level was observed in the adrenal glands during the first 28 d of anti-Thy 1.1 nephritis (data not shown). Despite these latter findings, a dramatic upregulation of PDGF-DD protein levels was detected in the serum of nephritic rats on day 8 after disease induction (27.7 ± 14.5 ng PDGF-DD/ml; $n = 9$) compared with the levels in normal animals that were consistently below the detection limit (<0.02 ng/ml; $n = 5$; data not shown).

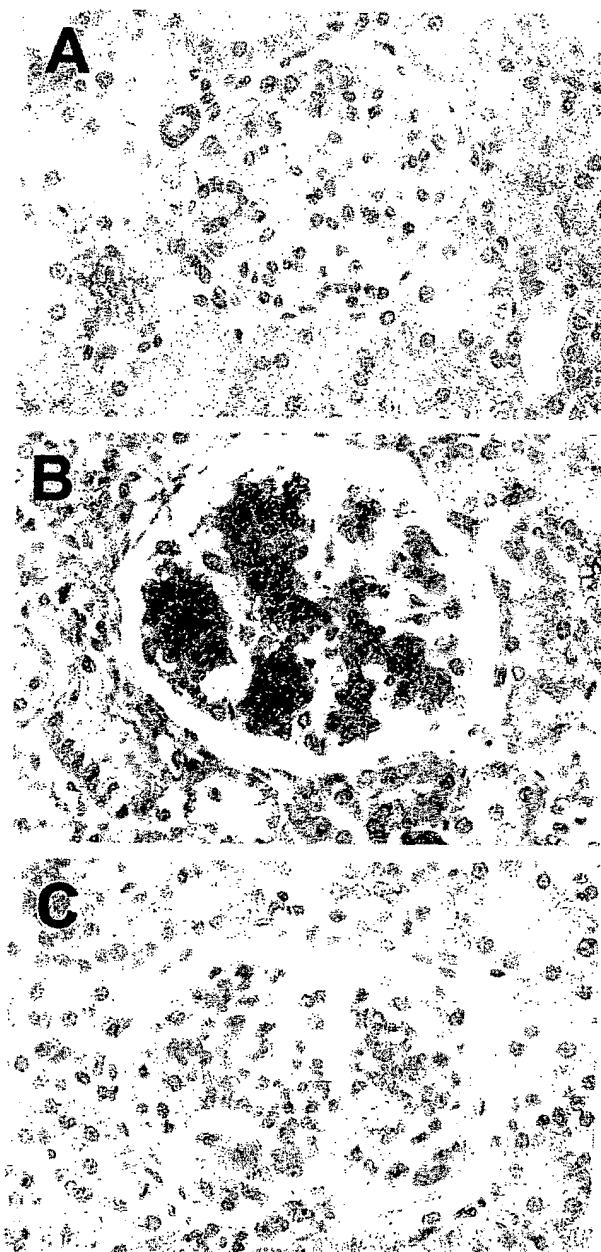
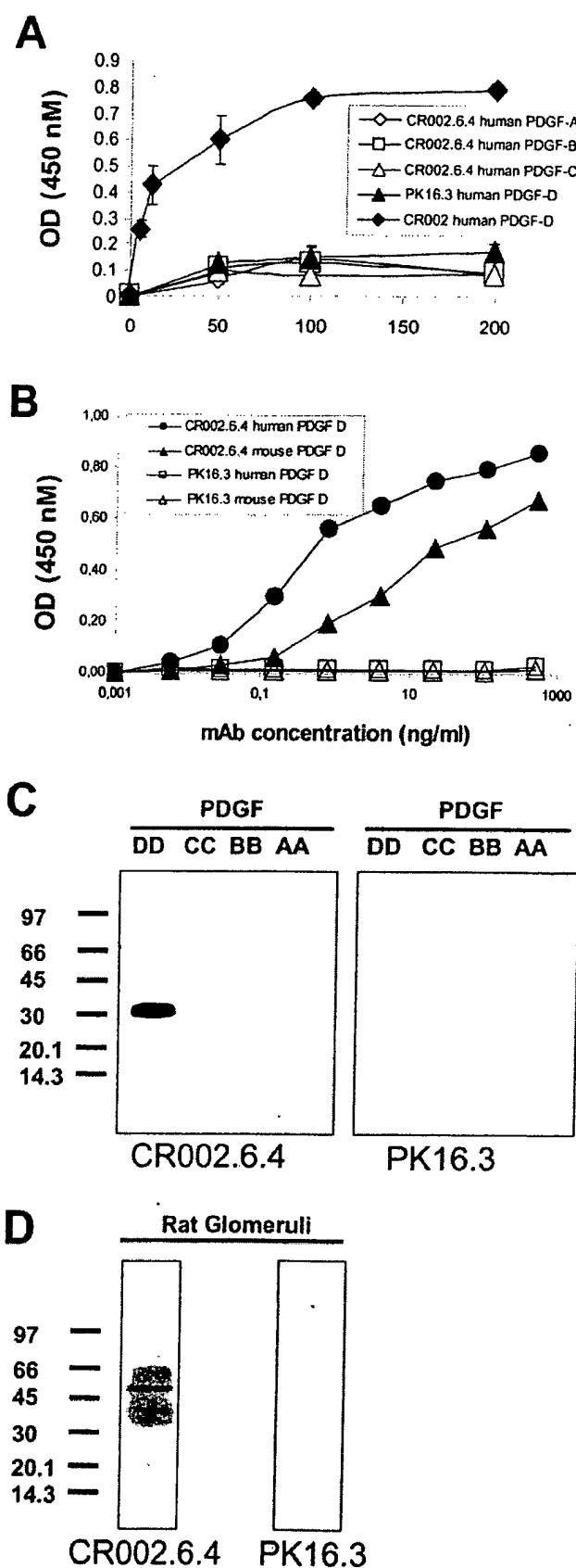


Figure 3. PDGF-DD is overexpressed during anti-Thy 1.1 nephritis in glomeruli. No PDGF-DD localization is noted in normal glomeruli (A), whereas mesangial expression can be readily detected during mesangiproliferative nephritis at day 7 after disease induction (B). No glomerular staining is present when the anti-PDGF-DD antibody is replaced by an equal concentration of control IgG (C). Magnification, $\times 600$.



By immunohistochemistry, PDGF-DD localization in normal rat kidney was confined to arterial and arteriolar vascular smooth muscle cells, whereas no immunoreactivity was noted in glomeruli (Figure 3A). During anti-Thy 1.1 nephritis, prominent glomerular localization of PDGF-DD in the expanded mesangium was present (Figure 3B), whereas the remaining staining pattern of the kidneys was not affected.

Characterization of the Fully Human PDGF-DD mAb CR002.6.4

The specificity of fully human mAb CR002.6.4 for PDGF-DD among the PDGF was characterized by both solid-phase ELISA and Western blot analysis. As shown in Figure 4, A and B, mAb CR002.6.4 recognized human and murine PDGF-DD but not human PDGF-AA, PDGF-BB, or PDGF-CC. Control mAb PK16.3 showed no recognition of PDGF-DD. To confirm the ELISA result, Western blot analysis was also performed. Figure 4C shows that mAb CR002.6.4 immunoblotted human PDGF-DD (p35) but not PDGF-AA, PDGF-BB, or PDGF-CC. As demonstrated in Figure 4D, mAb CR002.6.4 also immunoblotted rat PDGF-DD in glomerular lysates of rats with anti-Thy 1.1 nephritis on day 8 after disease induction. The observed differences in the size of bands between human recombinant PDGF-DD (approximately 35 kD) and PDGF-DD in rat glomerular protein lysates (approximately 50 kD) potentially reflect differences in posttranslational modifications and/or processing differences. Control mAb PK16.3 recognized no PDGF. BIACore kinetic measurements were used to determine that the affinity of CR002.6.4 for human PDGF-DD was 170 pM and that CR002.6.4 had an approximately 20-fold lower affinity for murine PDGF-DD (data not shown).

CR002.6.4 was next tested for its ability to neutralize human PDGF-DD-induced mitogenic activity in a NIH 3T3 BrdU incorporation assay. As shown in Figure 5, CR002.6.4 neutralized human PDGF-DD-induced BrdU incorporation with an IC_{50} of approximately 75 ng/ml. PDGF-BB-induced BrdU incorporation was not affected at the highest concentrations tested (5 μ g/ml; data not shown). Control mAb did not affect PDGF-DD-induced BrdU incorporation. CR002.6.4 also neutralized murine PDGF-DD-induced BrdU incorporation in normal human lung fibroblasts with an IC_{50} of approximately 250 ng/ml (data not shown). Concerning this, it is to stress that rat

Figure 4. Characterization of CR002.6.4 specificity by ELISA and Western blot analysis. (A and B) ELISA. Microtiter plates coated with human PDGF-AA, PDGF-BB, PDGF-CC, or PDGF-DD (A) and with human or murine PDGF-DD (B) were incubated with the indicated concentration of PDGF-DD mAb CR002.6.4 or control mAb PK16.3 as described in the Materials and Methods section. (C and D) Western blot analysis. Nonreducing SDS-PAGE was performed after loading gels with 250 ng of human PDGF-AA, PDGF-BB, PDGF-CC, or PDGF-DD (C) or with 20 μ g of rat glomerular protein lysates (D). After transfer to Hybond-P membranes, filters were treated with PDGF-DD mAb CR002.6.4 or control mAb PK16.3 as described in the Materials and Methods section.

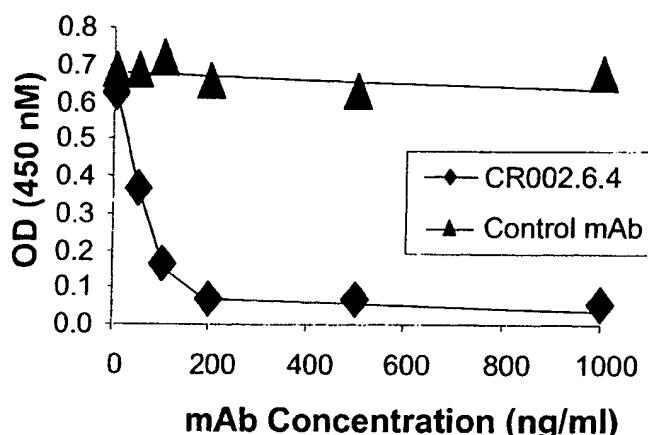


Figure 5. Neutralization of PDGF-DD-induced BrdU incorporation by CR002.6.4. NIH 3T3 fibroblasts were serum-starved and then incubated with PDGF-DD for 18 h in the presence of the indicated concentration of mAb CR002.6.4 or control. BrdU incorporation was measured as described in the Materials and Methods section. A representative experiment is presented.

and mouse PDGF-D are virtually identical. There is only one conservative amino acid substitution in the relevant core domain (L in rat to V in mouse) demonstrating homology of at least 99% between both species. This high homology and the specific recognition of rat PDGF-DD by CR002.6.4 (Figure 4D) strongly pled for a similar neutralization capacity of CR002.6.4 for rat PDGF-DD-induced mitogenic activity. Taken together, these results demonstrate that CR002.6.4 is highly specific for PDGF-DD, does not recognize other PDGF

family members, and potently neutralizes PDGF-DD-induced BrdU incorporation.

Inhibition of PDGF-DD In Vivo Reduces Pathologic Mesangial Cell Proliferation, Glomerular Matrix Accumulation, and Glomerular Monocyte/Macrophage Influx

After the injection of anti-Thy 1.1 mAb, PBS-treated animals developed the typical course of nephritis, which is characterized by early mesangiolysis and is followed by a phase of mesangial cell proliferation and matrix accumulation on days 5 and 8. No obvious adverse effects were noted after the repeated injection of CR002.6.4 (all rats survived and seemed normal until the end of the study). Serum levels of the mAb that were achieved in the nephritic groups are shown in Table 2. Measurement of the total hemolytic activity (classic complement pathway) and hemolytic activity of the alternative complement pathway in rat sera with and without human mAb levels revealed normal hemolytic activity in all sera ($CH_{50} < 284$ kU/L; $AP_{50} < 38\%$ of normal range), suggesting no effects of the human IgG2 on complement fixation.

A considerable increase in albuminuria was present on day 7 in the nephritic as compared with nonnephritic rats (albumin/creatinine ratio, 15.5 ± 5.6 mg/ μ mol in nephritic rats receiving PBS versus 0.3 ± 0.1 mg/ μ mol in nonnephritic rats receiving control mAb PK16.3; $P < 0.01$). No significant differences were noted between the nephritic groups receiving PBS, control mAb PK16.3, or the three doses of CR002.6.4 (Table 2). CR002.6.4 did not induce proteinuria in normal rats. This absent effect of PDGF-DD antagonism on proteinuria in the anti-Thy 1.1 nephritis model is similar to observations made

Table 2. Human CR002.6.4 mAb or irrelevant control PK16.3 mAb levels achieved *in vivo*, urinary albumin/creatinine, and systolic BP^a

Groups	Human mAb Serum Level (μ g/ml) Day 5 after Disease Induction	Human mAb Serum Level (μ g/ml) Day 8 after Disease Induction	Urinary Albumin/Creatinine Ratio (mg/ μ mol) Day 7 after Disease Induction	Systolic BP (mmHg) Day 7 after Disease Induction
Nephritic + CR002.6.4 5 mg/kg (day 3) + 2 mg/kg (day 5)	42 ± 9 ($n = 7$)	39 ± 26 ($n = 7$)	17.9 ± 9.4 ($n = 7$)	112 ± 11 ($n = 3$)
Nephritic + CR002.6.4 10 mg/kg (day 3) + 4 mg/kg (day 5)	75 ± 29 ($n = 7$)	65 ± 36 ($n = 7$)	18.0 ± 6.7 ($n = 7$)	136 ± 7 ($n = 3$)
Nephritic + CR002.6.4 20 mg/kg (day 3) + 8 mg/kg (day 5)	188 ± 85 ($n = 8$)	112 ± 72 ($n = 8$)	20.5 ± 23.3 ($n = 8$)	131 ± 21 ($n = 4$)
Nephritic + PK16.3 20 mg/kg (day 3) + 8 mg/kg (day 5)	134 ± 29 ($n = 8$)	95 ± 47 ($n = 8$)	15.7 ± 4.7 ($n = 8$)	119 ± 7 ($n = 4$)
Nephritic + PBS (day 3 and day 5)	<0.02 ($n = 9$)	<0.02 ($n = 9$)	15.5 ± 5.6 ($n = 9$)	132 ± 15 ($n = 5$)
Normal + CR002.6.4 10 mg/kg (day 3) + 4 mg/kg (day 5)	ND	ND	0.2 ± 0.3 ($n = 5$)	111 ± 11 ($n = 3$)
Normal + PK16.3 10 mg/kg (day 3) + 4 mg/kg (day 5)	ND	ND	0.3 ± 0.1 ($n = 5$)	122 ± 8 ($n = 3$)

^a Data are mean values \pm SD. ND, not determined.

Albumin/creatinine ratios in nephritic groups are not significantly different. Systolic BP are not significantly different between all treatment groups. Day of mAb treatment indicated in parentheses.

previously in the same model with a PDGF-B antagonist (22). No significant effects of the various CR002.6.4 doses or of irrelevant control mAb PK16.3 on systemic BP levels were observed, and all animals remained close to the normal range on day 7 (Table 2).

Glomerular cell proliferation, as assessed by counting the number of glomerular mitoses, was significantly reduced on day 8 in rats that received the anti-PDGF-D mAb CR002.6.4 as compared with rats that received control mAb PK16.3 or PBS ($P < 0.001$ for nephritic high-dose group *versus* nephritic controls; Figure 6). Counting of BrdU-positive nuclei confirmed these findings with the most pronounced suppression of proliferation on day 8 in the 20 + 8 mg/kg CR002.6.4-treated group ($P < 0.001$ for nephritic high-dose group *versus* nephritic controls; Figure 6).

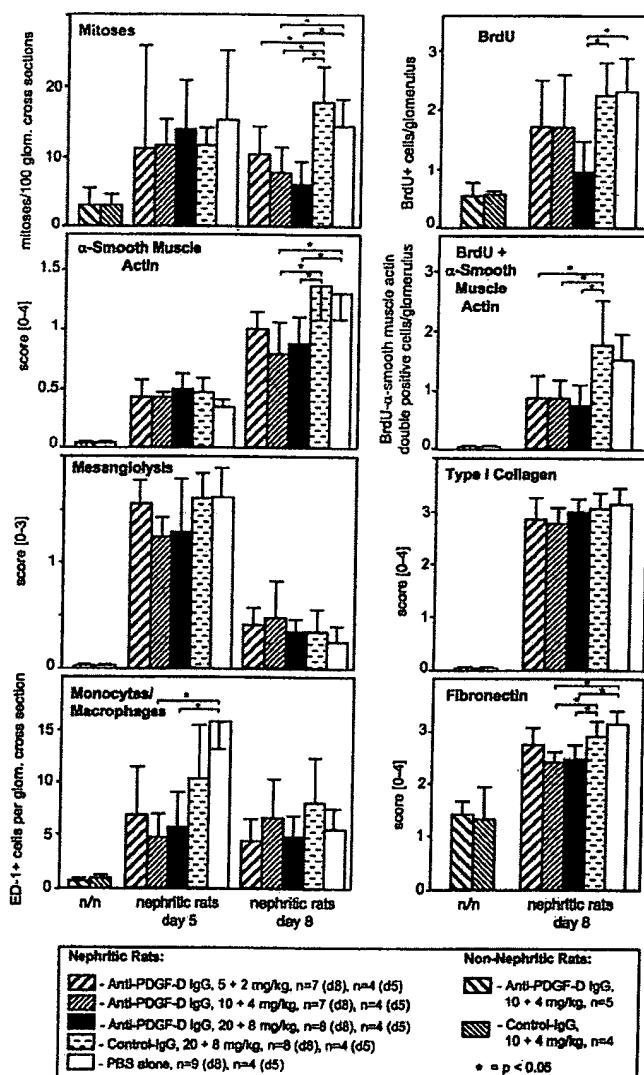


Figure 6. Glomerular changes on day 5 and day 8 after disease induction in rats with mesangiproliferative anti-Thy 1.1 nephritis treated with anti-PDGF-D CR002.6.4 mAb, control PK16.3 mAb, or PBS alone. Treatment was carried out on days 3 and 5. nn, nonnephritic rats treated with CR002.6.4 or PK16.3. * $P < 0.05$.

To assess the treatment effects on mesangial cells, we immunostained the renal sections for α -smooth muscle actin, which is expressed only by activated mesangial cells (32). The glomerular expression of α -smooth muscle actin was significantly reduced on day 8 in the rats that received 10 + 4 mg/kg ($P < 0.004$) and 20 + 8 mg/kg ($P < 0.005$) CR002.6.4 mAb as compared with rats that received control PK16.3 mAb or PBS (Figure 6). To determine specifically whether mesangial cell proliferation was reduced, we double immunostained CR002.6.4 mAb, PK16.3 mAb, or PBS-treated rats for BrdU and α -smooth muscle actin. The data confirmed a marked decrease of proliferating mesangial cells on day 8 after disease induction in all three CR002.6.4-treated groups with a maximum of 57% reduction in mesangial cell proliferation ($P < 0.009$ for nephritic high dose group *versus* nephritic controls; Figure 6). Injection of CR002.6.4 into normal rats did not affect the physiologic glomerular cell turnover as compared with normal rats that received irrelevant IgG (Figure 6).

The mesangiolysis scores were similar in CR002.6.4- and PK16.3-treated rats ($P = 0.41$ day 5 and $P = 0.92$ day 8 for nephritic high-dose group *versus* nephritic IgG control; Figure 6). In addition, immunostaining of glomeruli for human IgG (CR002.6.4 or PK16.3) showed identical intracapillary positivity but no deposition of either antibody in the expanded mesangium (data not shown), suggesting that anti-PDGF-D antibody CR002.6.4 did not bind to mesangial cells.

Glomerular accumulation of type I collagen was not affected by CR002.6.4 mAb treatment in any of the three nephritic groups compared with the rats that were treated with PK16.3 mAb or PBS alone (Figure 6). In contrast, treatment of the rats with either 10 + 4 or 20 + 8 mg/kg CR002.6.4 resulted in a reduction of glomerular fibronectin accumulation compared with the nephritic controls ($P < 0.004$ and $P < 0.01$ for the nephritic 10 + 4 and 20 + 8 mg/kg CR002.6.4 group *versus* nephritic controls, respectively; Figure 6). In normal rats, glomerular matrix expression was not affected by treatment with CR002.6.4 mAb or PK16.3 mAb ($P > 0.8$; Figure 6).

On day 5 but not day 8, all three doses of CR002.6.4 led to a marked reduction of glomerular monocyte/macrophage influx ($P < 0.003$ for nephritic high dose group *versus* nephritic PBS control on day 5; Figure 6). Treatment of nonnephritic rats with either the CR002.6.4 mAb or PK16.3 mAb had no effect on the glomerular monocyte/macrophage influx ($P = 0.23$; Figure 6).

Interactions of PDGF-BB and PDGF-DD

Given that both PDGF-BB and PDGF-DD are overproduced in anti-Thy 1.1 nephritis (Figures 2 and 3) and given that antagonism of either results in a reduction of mesangiproliferative changes, we assessed potential interactions of the two PDGF isoforms. Antagonism of PDGF-DD with CR002.6.4 had no significant effect on glomerular PDGF-B and PDGF-D mRNA levels on day 8 of the disease (Table 3).

Discussion

In this study, PDGF-DD was shown to induce proliferation in cultured rat and human mesangial cells. This observation is

Table 3. Glomerular PDGF-B and PDGF-D mRNA expression in CR002.6.4-treated rats^a

Groups	PDGF-B mRNA (Relative to Expression in Normal Rats + Control PK16.3)	PDGF-D mRNA (Relative to Expression in Normal Rats + Control PK16.3)
Nephritic + CR002.6.4 5 mg/kg (day 3) + 2 mg/kg (day 5)	1.60 (1.3-1.9) (n = 2)	1.90 (1.5-2.2) (n = 2)
Nephritic + CR002.6.4 10 mg/kg (day 3) + 4 mg/kg (day 5)	1.25 (1.0-1.6) (n = 3)	1.40 (1.2-1.5) (n = 3)
Nephritic + CR002.6.4 20 mg/kg (day 3) + 8 mg/kg (day 5)	1.35 (1.1-1.5) (n = 2)	1.45 (1.2-1.7) (n = 2)
Nephritic + PK16.3 20 mg/kg (day 3) + 8 mg/kg (day 5)	1.40 (1.1-1.7) (n = 3)	1.60 (1.4-1.7) (n = 3)
Nephritic + PBS (day 3 and day 5)	1.45 (1.1-1.8) (n = 3)	2.10 (1.5-2.6) (n = 3)
Normal + CR002.6.4 10 mg/kg (day 3) + 4 mg/kg (day 5)	0.95 (0.7-1.1) (n = 2)	1.10 (1.0-1.2) (n = 2)
Normal + PK16.3 10 mg/kg (day 3) + 4 mg/kg (day 5)	1.0 (n = 2)	1.0 (n = 2)

^a Data are means (and ranges) of pooled fractions within each treatment group. Measurements were performed twice for each sample. Day of mAb treatment indicated in parentheses.

consistent with previous data showing that mesangial cells in culture express both the PDGF receptor α - and β -chain (12). Furthermore, this study demonstrated intraglomerular overproduction and overexpression of PDGF-D-chain during the course of experimental mesangioproliferative nephritis. In contrast to most other cytokines and growth factors that act in a localized auto- or paracrine manner, PDGF-D in this model was notable for its dramatic increase in serum, *i.e.*, potential endocrine effects. In normal rat glomeruli, PDGF-D expression was absent, similar to recent findings on the expression of PDGF-C in rat kidney (7). In this respect, the species difference between rat and human kidney is notable, because both PDGF-C and -D are expressed in normal human podocytes, *i.e.*, glomerular visceral epithelial cells (6,7).

Given the above findings, we asked whether inhibition of PDGF-DD in the anti-Thy 1.1 mesangioproliferative nephritis model might result in a reduction of mesangioproliferative changes *in vivo*. In the anti-Thy 1.1 nephritis model, immunologic damage to the mesangium results in mesangiolysis, induces migration of nondamaged mesangial cells from the extraglomerular mesangium, and results in excessive proliferation leading to mesangioproliferative glomerulonephritis (38). In the present study, as in previous ones (22,25), we therefore initiated treatment on day 3 to avoid any interference of the therapy with disease induction, *e.g.*, glomerular binding of the nephritogenic antibody, complement activation, cytotoxic damage and mesangiolysis, all of which peak within 24 to 48 h of disease initiation. This timing was also chosen on the basis of glomerular PDGF-D mRNA kinetics that revealed an increase in PDGF-DD between days 2 and 4. Finally, this experimental design mimicked the clinical setting because treatment was initiated after the onset of mesangioproliferative glomerulonephritis.

Pharmacologic intervention with neutralizing PDGF-D mAb CR002.6.4 on days 3 and 5 after induction of anti-Thy 1.1 nephritis resulted in a significant dose-dependent reduction of excessive mesangial cell proliferation and thereby led to a marked reduction of glomerular hypercellularity. By double immunostaining, we were able to demonstrate that reduction of mesangial cell proliferation is a central contributor to the therapeutic effects observed. It seems unlikely that this was a

nonspecific effect, for example related to hemodynamic changes or effects on disease induction because CR002.6.4 mAb had no effect on systemic BP or mesangiolysis. Incidentally, the absent effect of the antibody on mesangiolysis additionally supports the idea of PDGF-D being a secreted rather than a membrane-bound protein (36) because enhanced mesangiolysis would have been the likely consequence if mAb CR002.6.4 were to bind to mesangial cells. The latter is also unlikely, because we failed to detect mesangial deposition of human IgG in rats that received the antibody. Finally, reduction of mesangial cell proliferation was specific for the CR002.6.4 mAb treatment.

Of further interest is whether CR002.6.4 mAb treatment directly reduced mesangial cell proliferation *in vivo* or, for example, via effects on other growth factors. Such cascades seem to be operative in anti-Thy 1.1 nephritis, as we have recently demonstrated that PDGF-BB most likely is a downstream mediator of TGF- β effects *in vivo* (39). A comparative evaluation of the mRNA expression of all four PDGF isoforms during anti-Thy 1.1 nephritis revealed that the peak of PDGF-D mRNA overproduction occurred after that of PDGF-B. Whether this is evidence of an induction of PDGF-D by PDGF-B *in vivo* remains unknown.

It is interesting that the glomerular fibronectin accumulation on day 8 was significantly reduced by PDGF-DD antagonism in the two high-dose groups, whereas glomerular type I collagen expression remained unchanged. These results point toward differences between PDGF-DD and PDGF-BB regulation of the glomerular matrix protein turnover because it has been demonstrated that PDGF-BB antagonism in experimental mesangioproliferative glomerulonephritis resulted in a significant reduction of both glomerular fibronectin and type I collagen (22).

In addition to selectively abrogating mesangial cell proliferation, treatment with the CR002.6.4 mAb, at least with higher doses, reduced the glomerular monocyte/macrophage influx on day 5. Whether this is affected directly or through modulation of other mediators is unknown. In this context, it seems noteworthy that monocyte chemoattractant protein-1 expression, which is known to be responsible for the glomerular infiltration of monocytes/macrophages in the early phase

of the anti-Thy 1.1 nephritis (40), is rapidly induced in fibroblasts upon stimulation with PDGF-BB (41).

In conclusion, this study provides the first evidence to implicate glomerular and systemic overexpression of PDGF-DD in the pathogenesis of mesangioproliferative changes, in particular enhanced mesangial cell proliferation. Thus, we demonstrate that PDGF-DD exerts the biologic effect *in vitro* and that the biologic effect *in vivo* is associated with overproduction and release of the factor. In addition, the *in vivo* effect can be diminished by specific antagonism of PDGF-DD. We thereby fulfill three of four criteria necessary for establishing a role for a growth factor in glomerular disease (42). The fourth criterion, namely “the effect is reproduced *in vivo* by administration or overexpression of the factor,” was recently reported in preliminary fashion by Hudkins *et al.* (43).

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References

1. Floege J, Ostendorf T, Wolf G: Growth factors and cytokines. In: *Immunologic Renal Diseases*, 2nd Ed., edited by Neilson EG, Couser WG, Philadelphia, Lippincott-Raven, 2001, pp 415–463
2. LaRochelle WJ, Jeffers M, McDonald WF, Chillakuru RA, Giese NA, Lokker NA, Sullivan C, Boldog FL, Yang M, Vernet C, Burgess CE, Fernandes E, Deegler LL, Rittman B, Shimkets J, Shimkets RA, Rothberg JM, Lichenstein HS: PDGF-D, a new protease-activated growth factor. *Nat Cell Biol* 3: 517–521, 2001
3. Li X, Ponten A, Aase K, Karlsson L, Abramsson A, Uutela M, Backstrom G, Hellstrom M, Bostrom H, Li H, Soriano P, Betsholtz C, Heldin CH, Alitalo K, Ostman A, Eriksson U: PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor. *Nat Cell Biol* 2: 302–309, 2000
4. Bergsten E, Uutela M, Li X, Pietras K, Ostman A, Heldin CH, Alitalo K, Eriksson U: PDGF-D is a specific, protease-activated ligand for the PDGF beta-receptor. *Nat Cell Biol* 3: 512–516, 2001
5. Gilbertson DG, Duff ME, West JW, Kelly JD, Sheppard PO, Hofstrand PD, Gao Z, Shoemaker K, Bukowski TR, Moore M, Feldhaus AL, Humes JM, Palmer TE, Hart CE: Platelet-derived growth factor C (PDGF-C), a novel growth factor that binds to PDGF alpha and beta receptor. *J Biol Chem* 276: 27406–27414, 2001
6. Changsirikulchai S, Hudkins KL, Goodpaster TA, Volpone J, Topouzis S, Gilbertson DG, Alpers CE: Platelet-derived growth factor-D expression in developing and mature human kidneys. *Kidney Int* 62: 2043–2054, 2002
7. Eitner F, Ostendorf T, Van Roeyen C, Kitahara M, Li X, Aase K, Grone HJ, Eriksson U, Floege J: Expression of a novel PDGF isoform, PDGF-C, in normal and diseased rat kidney. *J Am Soc Nephrol* 13: 910–917, 2002
8. Striker LJ, Peten EP, Elliot SJ, Doi T, Striker GE: Mesangial cell turnover: Effect of heparin and peptide growth factors. *Lab Invest* 64: 446–456, 1991
9. Abboud HE, Poptic E, DiCorleto PE: Production of platelet-derived growth factor-like protein by rat mesangial cells in culture. *J Clin Invest* 80: 675–683, 1987
10. Shultz PJ, DiCorleto PE, Silver BJ, Abboud HE: Mesangial cells express PDGF mRNAs and proliferate in response to PDGF. *Am J Physiol* 255: F674–F684, 1988
11. Silver BJ, Jaffer FE, Abboud HE: Platelet-derived growth factor synthesis in mesangial cells: Induction by multiple peptide mitogens. *Proc Natl Acad Sci U S A* 86: 1056–1060, 1989
12. Floege J, Topley N, Hoppe J, Barrett TB, Resch K: Mitogenic effect of platelet-derived growth factor in human glomerular mesangial cells: Modulation and/or suppression by inflammatory cytokines. *Clin Exp Immunol* 86: 334–341, 1991
13. Waldherr R, Noronha IL, Niemir Z, Kruger C, Stein H, Stumm G: Expression of cytokines and growth factors in human glomerulonephritides. *Pediatr Nephrol* 7: 471–478, 1993
14. Gesualdo L, Pinzani M, Floriano JJ, Hassan MO, Nagy NU, Schena FP, Emancipator SN, Abboud HE: Platelet-derived growth factor expression in mesangial proliferative glomerulonephritis. *Lab Invest* 65: 160–167, 1991
15. Gesualdo L, Di Paolo S, Milani S, Pinzani M, Grappone C, Ranieri E, Pannarale G, Schena FP: Expression of platelet-derived growth factor receptors in normal and diseased human kidney. An immunohistochemistry and *in situ* hybridization study. *J Clin Invest* 94: 50–58, 1994
16. Fellstrom B, Klareskog L, Heldin CH, Larsson E, Ronnstrand L, Terracio L, Tufveson G, Wahlberg J, Rubin K: Platelet-derived growth factor receptors in the kidney—Upregulated expression in inflammation. *Kidney Int* 36: 1099–1102, 1989
17. Yoshimura A, Inui K, Suzuki T: Demonstration of PDGFB-chain mRNA in the cellular crescent in human glomerular diseases. *J Am Soc Nephrol* 4: 692, 1993
18. Floege J, Eng E, Young BA, Alpers CE, Barrett TB, Bowen-Pope DF, Johnson RJ: Infusion of platelet-derived growth factor or basic fibroblast growth factor induces selective glomerular mesangial cell proliferation and matrix accumulation in rats. *J Clin Invest* 92: 2952–2962, 1993
19. Isaka Y, Fujiwara Y, Ueda N, Kaneda Y, Kamada T, Imai E: Glomerulosclerosis induced by *in vivo* transfection of transforming growth factor-beta or platelet-derived growth factor gene into the rat kidney. *J Clin Invest* 92: 2597–2601, 1993
20. Leveen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E, Betsholtz C: Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev* 8: 1875–1887, 1994
21. Soriano P: Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes Dev* 8: 1888–1896, 1994
22. Floege J, Ostendorf T, Janssen U, Burg M, Radeke HH, Vargeese C, Gill SC, Green LS, Janjic N: Novel approach to specific growth factor inhibition *in vivo*: Antagonism of platelet-derived growth factor in glomerulonephritis by aptamers. *Am J Pathol* 154: 169–179, 1999
23. Gilbert RE, Kelly DJ, McKay T, Chadban S, Hill PA, Cooper ME, Atkins RC, Nikolic-Paterson DJ: PDGF signal transduction inhibition ameliorates experimental mesangial proliferative glomerulonephritis. *Kidney Int* 59: 1324–1332, 2001
24. Nakamura H, Isaka Y, Tsujie M, Akagi Y, Sudo T, Ohno N, Imai E, Hori M: Electroporation-mediated PDGF receptor-IgG chimera gene transfer ameliorates experimental glomerulonephritis. *Kidney Int* 59: 2134–2145, 2001
25. Ostendorf T, Kunter U, Grone HJ, Bahlmann F, Kawachi H, Shimizu F, Koch KM, Janjic N, Floege J: Specific antagonism of PDGF prevents renal scarring in experimental glomerulonephritis. *J Am Soc Nephrol* 12: 909–918, 2001

26. Kliem V, Johnson RJ, Alpers CE, Yoshimura A, Couser WG, Koch KM, Floege J: Mechanisms involved in the pathogenesis of tubulointerstitial fibrosis in 5/6-nephrectomized rats. *Kidney Int* 49: 666–678, 1996
27. Tang WW, Ulich TR, Lacey DL, Hill DC, Qi M, Kaufman SA, Van GY, Tarpley JE, Yee JS: Platelet-derived growth factor-BB induces renal tubulointerstitial myofibroblast formation and tubulointerstitial fibrosis. *Am J Pathol* 148: 1169–1180, 1996
28. Yang XD, Corvalan JR, Wang P, Roy CM, Davis CG: Fully human anti-interleukin-8 monoclonal antibodies: Potential therapeutics for the treatment of inflammatory disease states. *J Leukoc Biol* 66: 401–410, 1999
29. Green LS, Jellinek D, Jenison R, Ostman A, Hedin CH, Janjic N: Inhibitory DNA ligands to platelet-derived growth factor B-chain. *Biochemistry* 35: 14413–14424, 1996
30. Radeke HH, Gessner JE, Uciechowski P, Magert HJ, Schmidt RE, Resch K: Intrinsic human glomerular mesangial cells can express receptors for IgG complexes (hFc gamma RIII-A) and the associated Fc epsilon RI gamma-chain. *J Immunol* 153: 1281–1292, 1994
31. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159, 1987
32. Johnson RJ, Iida H, Alpers CE, Majesky MW, Schwartz SM, Pritzl P, Gordon K, Gown AM: Expression of smooth muscle cell phenotype by rat mesangial cells in immune complex nephritis. Alpha-smooth muscle actin is a marker of mesangial cell proliferation. *J Clin Invest* 87: 847–858, 1991
33. Burg M, Ostendorf T, Mooney A, Koch KM, Floege J: Treatment of experimental mesangioproliferative glomerulonephritis with non-anticoagulant heparin: Therapeutic efficacy and safety. *Lab Invest* 76: 505–516, 1997
34. Johnson RJ, Garcia RL, Pritzl P, Alpers CE: Platelets mediate glomerular cell proliferation in immune complex nephritis induced by anti-mesangial cell antibodies in the rat. *Am J Pathol* 136: 369–374, 1990
35. Yoshimura A, Gordon K, Alpers CE, Floege J, Pritzl P, Ross R, Couser WG, Bowen-Pope DF, Johnson RJ: Demonstration of PDGF B-chain mRNA in glomeruli in mesangial proliferative nephritis by in situ hybridization. *Kidney Int* 40: 470–476, 1991
36. LaRochelle WJ, Jeffers M, Corvalan JR, Jia XC, Feng X, Vaneegas S, Vickroy JD, Yang XD, Chen F, Gazit G, Mayotte J, Macaluso J, Rittman B, Wu F, Dhanabal M, Herrmann J, Lichenstein HS: Platelet-derived growth factor D: Tumorigenicity in mice and dysregulated expression in human cancer. *Cancer Res* 62: 2468–2473, 2002
37. Kitahara M, Eitner F, Ostendorf T, Kunter U, Janssen U, Westenfeld R, Matsui K, Kerjaschki D, Floege J: Selective cyclooxygenase-2 inhibition impairs glomerular capillary healing in experimental glomerulonephritis. *J Am Soc Nephrol* 13: 1261–1270, 2002
38. Hugo C, Shankland SJ, Bowen-Pope DF, Couser WG, Johnson RJ: Extraglomerular origin of the mesangial cell after injury. A new role of the juxtaglomerular apparatus. *J Clin Invest* 100: 786–794, 1997
39. Ostendorf T, Kunter U, van Roeyen C, Dooley S, Janjic N, Ruckman J, Eitner F, Floege J: The effects of platelet-derived growth factor antagonism in experimental glomerulonephritis are independent of the transforming growth factor-beta system. *J Am Soc Nephrol* 13: 658–667, 2002
40. Wenzel U, Schneider A, Valente AJ, Abboud HE, Thaiss F, Helmchen UM, Stahl RA: Monocyte chemoattractant protein-1 mediates monocyte/macrophage influx in anti-thymocyte antibody-induced glomerulonephritis. *Kidney Int* 51: 770–776, 1997
41. Yoshimura T, Leonard EJ: Secretion by human fibroblasts of monocyte chemoattractant protein-1, the product of gene JE. *J Immunol* 144: 2377–2383, 1990
42. Johnson RJ, Floege J, Couser WG, Alpers CE: Role of platelet-derived growth factor in glomerular disease. *J Am Soc Nephrol* 4: 119–128, 1993
43. Hudkins KL, Gilbertson DG, Hughes SE, Holden M, Palmer TE, Feldhaus AL, Alpers CE: Mesangial proliferative glomerulopathy induced by PDGF-D resulting from adenovirus mediated gene transfer [Abstract]. *J Am Soc Nephrol* 13: 132A, 2002

The Human Immunoglobulin Genes

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Introduction

The results that I will describe here concern the structure and rearrangement of the genes coding for antibodies in man. A great deal of work was conducted in several laboratories on the analogous genes of mouse. I shall make no attempt to review this work. The human antibody gene system was chosen for study largely because of the existence of a wide range of defined, clinical disorders of the immune system, which include the immunodeficiency diseases (e.g. agammaglobulinaemia) and the leukaemias [frequently tumours of immunoglobulin (Ig) producing cells]. In the preparation and analysis of molecular probes for the immunoglobulin genes, we have gained some knowledge of the arrangement and rearrangement of these genes in man and it is clear that the human antibody genes represent a fluid system which employs a number of highly developed DNA rearrangement procedures in their activation.

The antibody proteins are made up from two types of polypeptide chain, the heavy (H) and light (L) chains. Each polypeptide itself has an N-terminal variable (V) region and a C-terminal constant (C) region. The V-region of the heavy (V_H) and light (V_L) chains together make the antibody combining site capable of specific antigen recognition (defined by the amino-acid sequence of V-regions). The C-region, particularly that of the H-chain (C_H), performs more constant functions such as complement fixation. It is the C_H -region which defines the antibody class; there are five classes of C_H -sequence called μ , δ , γ , ϵ or α (giving IgM, IgD, IgG, IgE and IgA respectively). The C_H -region sequence is invariant in each class except γ and α where amino-acid differences define γ_1 , γ_2 , γ_3 or γ_4 and α_1 or α_2 (giving IgG1, 2, 3 or 4 and IgA1 or 2 respectively). Any of the H-chain classes or subclasses can associate with either of the two types of L-chain, κ - or λ -chains (again defined by their respective amino-acid sequences).

During the development of a lymphocyte the first gene to be expressed is μ followed by L-chain induction resulting in the formation of surface IgM (Cooper *et al.* 1976; Knapp *et al.* 1973; Pernis *et al.*, 1976). A B-lymphocyte clone can subsequently initiate IgD production, in addition to the IgM it already makes; the two heavy chains involved here (i.e. μ and δ) express the same V_H -segment. A subsequent event occurs, the H-chain class switch, which results in the expression of IgG, IgA or IgE in place of IgM and IgD but maintaining the same antibody combining site (Sledge *et al.* 1976; Wang *et al.* 1970). These various events involve a complex set of chromosomal DNA rearrangements. Basically in the germ-line the antibody genes are in pieces and the fully active gene is created, within the B-cells, by these rearrangements.

Chromosomal mapping of the human Ig genes and association to sites of translocation in malignant lymphoid cells

The chromosomal assignments of the H- and L-chain genes have been made by a variety of methods and the results of our studies and those of other laboratories are summarized in Table 1. We showed that V_H - and C_H -genes both reside in chromosome 14 by using Southern filter hybridization techniques (Southern, 1975) to analyse the presence of H-chain genes in mouse x human somatic cell hybrids which have a defined but incomplete human karyotype (Hobart *et al.* 1981). Fig. 1 is a diagram of this analysis in which positively hybridizing clones

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were scored and the data clearly show that the H-chain genes occur on chromosome 14.

In accord with the work of Erikson *et al.* (1981) showing that chromosome 22 carries the λ light chain locus, a clone library had been prepared from chromosome 22 which had been

Table 1. Chromosomal mapping of human immunoglobulin genes

Gene locus	Assigned chromosome	Reference
κ -chain	2	Malcolm <i>et al.</i> 1982 McBride <i>et al.</i> 1982
λ -chain	22	Erikson <i>et al.</i> 1981 McBride <i>et al.</i> 1982
H-chain	14	Rabbits <i>et al.</i> 1982 Croce <i>et al.</i> 1979 Hobart <i>et al.</i> 1981 Kirsch <i>et al.</i> 1982

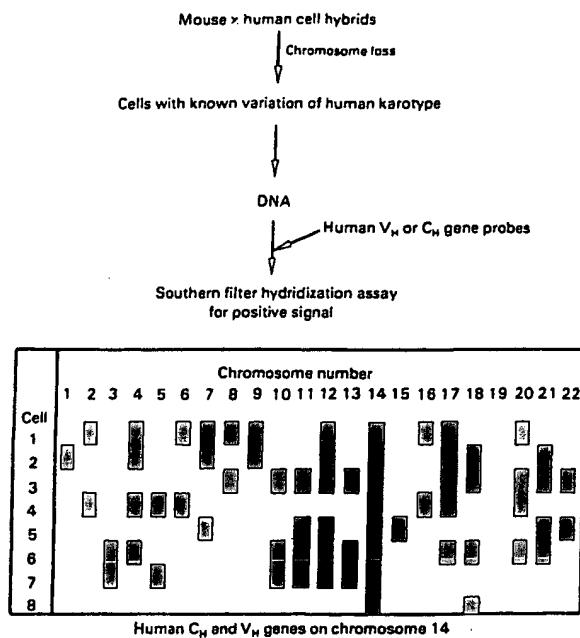


Fig. 1. Chromosomal localization of human heavy chain genes

fractionated in the fluorescence activated cell sorter (Krumlauf *et al.*, 1982) and this library was used to isolate both V_{λ} and C_{λ} genes (T. H. Rabbits, F. T. Kao & B. Young, unpublished work); the proof of concordance between these λ -genes and the long arm of chromosome 22 was made by showing a selective loss of the relevant genes in cell-hybrids with progressive deletions of the long arm of chromosome 22 (T. H. Rabbits, F. T. Kao & B. Young, unpublished work).

A completely different approach was used to localize the κ -chain genes to chromosome 2. This was the method of *in situ* hybridization using cloned V_{κ} probes (Malcolm *et al.* 1982). The hybridization of two independent genomic V_{κ} clones to metaphase spreads of phytohaemagglutinin-stimulated T-lymphocytes or to fibroblast cultures showed a specific localization of grains to chromosome 2. The specificity of such hybridization can be shown in a variety of ways but one in which the gene localization is more clearly established is to study the hybridization to cells carrying balanced reciprocal translocations. Fig. 2 shows the results of V_{κ} -probe hybridization to a cell carrying a 2;16 translocation [46XXt(2;16)(q13;q22)]. The specific hybridization signal was observed on the normal chromosome 2 and in the 2/16 chromosome, whilst no signal could be seen in association with either 16/2 or the normal 16 chromosome. The hybridization to chromosome 2/16 further helps to localize the κ -genes within either the short arm or the section of the long arm between q13 and the centromere of chromosome 2. In fact an analysis of the grain distribution over all the normal chromosomes 2 in 106 karyotypes enabled us to localize the V_{κ} hybridization to the short arm near the centromere (2p12).

The localization of the three immunoglobulin genes to the three separate autosomes 2 (κ), 14 (H) and 22 (λ) has some significant implications for some specific chromosome translocations identified in the malignant lymphoid tumours, Burkitt lymphoma and chronic myeloid leukaemia. Many Burkitt lymphoma cells show a specific 8;14 translocation (Klein, 1981) whilst other variant cells show either 2;8 or 8;22 translocations (Bernheim *et al.*, 1981). Furthermore, greater than 90% of chronic myeloid leukaemia patients possess Ph⁺ positive cells which seem to be reciprocal translocation of

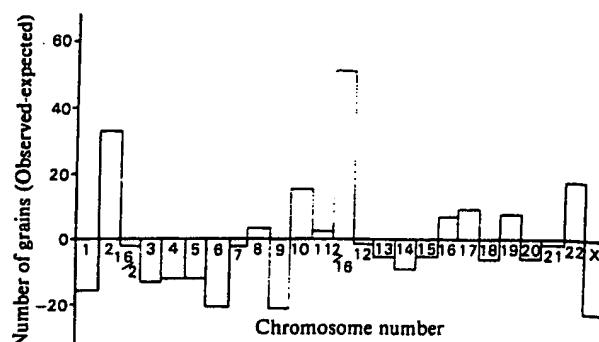


Fig. 2. *In situ* hybridization of [³H]cRNA, made on the HK101 gene, to translocated (2;16) chromosomes

The number of grains observed over all chromosomes from 22 karyotyped cells hybridized to HK101 is shown. The expected value for grains is calculated by dividing total grains counted by the relative chromosome size.

chromosomes 22 and 9 (Rowley, 1982). It is interesting that in Burkitt lymphoma each of the Ig gene-containing chromosomes can be involved in a specific translocation of apparently the same segment of chromosome 8 and also that the break in the chromosome 22 of chronic myeloid leukaemia is related to the analogous Burkitt's lymphoma break.

The specificity of the chromosomal translocations in these malignant tumours may well be coupled to the fact that the break points are associated in some way with the Ig genes which are known to be undergoing rearrangements during their activation. This raises two possibilities. Firstly that the translocation in itself is an event which contributes to the cell transformation and secondly that Ig gene rearrangement together with related sequences are responsible for the occasional inter-chromosomal event which has such catastrophic consequences. In the remainder of this paper, the different types of DNA rearrangements and the associated sequences which occur in the human Ig locus will be discussed.

The structure and origin of diversity of human V -genes

The germ-line DNA contains separate V - and C -region genes which join in the expressing cells (Hozumi & Tonegawa, 1976; Rabbits & Forster, 1978). Isolated V_{κ} - and V_{λ} -genes have a structure outlined in Fig. 3. Both types of V -gene have a 5' leader sequence interrupted at the codon -5 by an intervening sequence of about 100 nucleotides (Matthyssens & Rabbits, 1980; Bentley & Rabbits, 1980). The remainder of the sequence is uninterrupted coding region. Both genes end prematurely in the germ-line compared with the V -region as defined by the protein sequence. This excluded portion of the V -region is the J -region (and also D -region in H -chains) which lies adjacent to the C -gene (see the next section).

The portion of the antibody which recognises antigen is the V -region, so that a multiplicity of different V -gene sequences are required. How are these sequences generated? One component of the diversity of V -genes is undoubtedly the presence of multiple V -genes in the germ-line. This can simply be demonstrated by Southern filter hybridization analysis of nuclear DNA using V -gene probes. An example of this is shown in Fig. 4 in which the hybridization probe was derived from the $V_{\kappa}I$ subgroup. Each of eight unrelated DNAs displayed about 15 hybridization bands with evidence of some genetic polymorphism. V_{λ} probes used in analogous experiments showed similar patterns of bands although the complexity of the hybridization observed was greater (Matthyssens & Rabbits, 1980). When the hybridization properties of probes from two

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Precursor
V-segments

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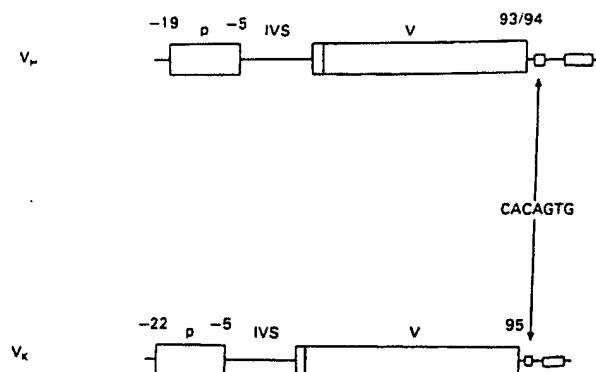
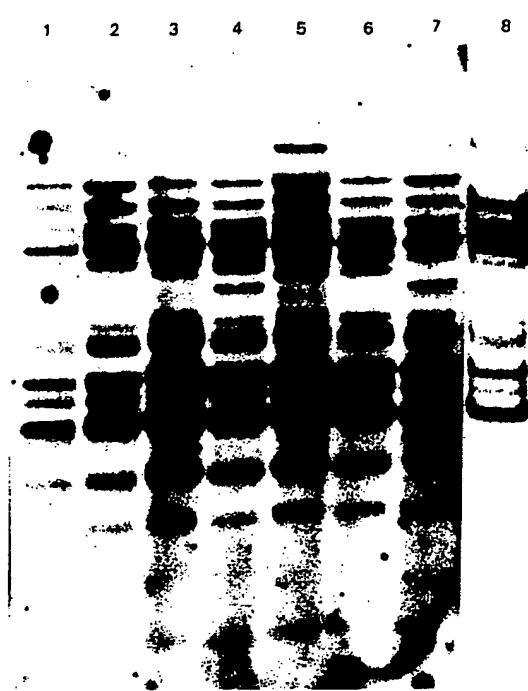


Fig. 3. Structure of human V-genes

V_{κ} and V_{μ} -genes were isolated from foetal liver DNA. p, Precursor or leader sequence; IVS, intervening sequence; V, V-region or V-gene.

Fig. 4. Southern filter hybridization patterns of V_{κ} -genes from eight unrelated humans

A V_{κ} I probe was hybridized with Bgl II-digested DNA from: slot 1, 160 cell-line (Karpas *et al.*, 1977); slot 2, MOLT4 cell-line (Minowada *et al.*, 1972); slot 3, Daudi cell-line (Klein *et al.*, 1968); slot 4, HeLa cells; slot 5, spleen; slot 6, placenta; slot 7, placenta; slot 8, foetal liver.

different human V_{κ} subgroups were compared, we found very little difference in the patterns of hybridization (Bentley & Rabbits, 1981) indicating that we were detecting, with a given probe, a large proportion of the V_{κ} -gene pool. This conclusion has implications for the origin of V -gene diversity in man. The size of the V_{κ} -gene pool based on these estimates is likely to be of the order of 20–30 different V -genes per chromosome. If we assume that allelic variation is widespread we can double these figures (i.e. 40–60). This limited number of V_{κ} -genes indicates that somatic variation is a major contributor to the total V_{κ}

repertoire in man. The arguments supporting such a conclusion have been discussed (Bentley & Rabbits, 1981) so will not be repeated here.

Joining segments of the human H -chain locus

In addition to the mutational events alluded to above there is a combinatorial diversity [first described for mouse κ -chains by the laboratories of Leder and Tonegawa (Max *et al.*, 1979; Sakano *et al.*, 1979)] generated by the V -gene joining which occurs during the active gene formation. As mentioned above, both V_{κ} - and V_{μ} -genes in the germ-line do not encode the full V -region as defined by comparative protein sequencing of many V -regions. This deficit is made up in part from a set of J_{μ} or joining segments located upstream of the C_{μ} -gene. We identified four J_{μ} and a pseudo (ψ) J_{μ} segment about 8000 bases upstream of the 5' end of the human μ -gene. Two further J_{μ} -regions have been identified (Ravetch *et al.*, 1981). The sequence and arrangement of these is shown in Fig. 5 where the region encoding J_{μ} , C_{μ} and C_{δ} is indicated as deduced from two overlapping phage clones derived from human foetal liver DNA. The DNA which comes between the various J_{μ} segments (about 300 bases) and between the J_{μ} - and μ -gene is intervening sequence which is post-transcriptionally removed. Similarly the μ -gene, in keeping with all C_{μ} -genes in mouse and man, has genetic domains (reflecting the protein domains) separated by short intervening sequences (around 300 bases in length). The human μ -gene, like the κ -gene (Rabbits *et al.*, 1981; Flanagan & Rabbits, 1982a), but unlike the γ -genes (Krawinkel & Rabbits, 1982; Ellison & Hood, 1982; Takahashi *et al.*, 1982), possesses four distinct domains and lacks a hinge segment. In the human γ -genes, each has a single hinge segment between $C_{\gamma}1$ and $C_{\gamma}2$ domains; the exception is the γ_3 -gene which has four separate hinge segments lying between $C_{\gamma}1$ and $C_{\gamma}2$ (Krawinkel & Rabbits, 1982; Takahashi *et al.*, 1982).

Whereas the V_{μ} -gene segment has codons 1–93 the J_{μ} -segment only carries the codons 100–113. This means that even including these segments a small piece of V -region is missing: this segment is the D-segment (first postulated in the mouse by Early *et al.*, 1980a). Such D-segments have subsequently been identified in human DNA (Siebenlist *et al.*, 1981) making a complete picture of the V_{μ} -gene integration possible; this is diagrammatically shown in Fig. 6. One of a set of V_{μ} -segments joins to one [or possibly more (Kurosawa & Tonegawa, 1982)] D-segments followed by joining to a J_{μ} -segment. These multiple chromosomal rearrangements lead to the formation of the active C_{μ} -gene. The cells are now able to make μ -chains which, of course, come from a transcript containing V_{μ} and C_{μ} on a single pre-mRNA molecule. RNA splicing subsequently removes transcribed intervening sequences (Rabbits, 1978) resulting in the mRNA from which heavy chain protein is translated.

The events described above indicate that a remarkable set of DNA rearrangement events take place which lead to the transcription of the active μ -gene in association with the integrated V -gene. One of the puzzles of this gene activation is that in the B-lymphocyte the germ-line V -genes are not transcribed (Mather & Perry, 1981). This indicates a very powerful mechanism which selects for the integrated gene. Such a mechanism could be simply at the level of DNA sequence; for example, new sequences could be created or old ones removed after V -gene joining, thereby creating an active promoter. An alternative possibility is that all germ-line V -genes carry a promoter sequence (which is transported with the gene during integration) but that the non-integrated genes are masked from transcription (e.g. by the chromatin structure). In relation to these possibilities, we asked whether or not germ-line V_{κ} -genes carry a promoter sequence and the answer seems to be that they do (Bentley *et al.*, 1982). We have utilized cloned V_{κ} -genes from human foetal liver (i.e. unintegrated, non-expressed V -genes) to study their ability to undergo transcription in either an *in vitro* system prepared from HeLa cells or *in vivo* after injection into

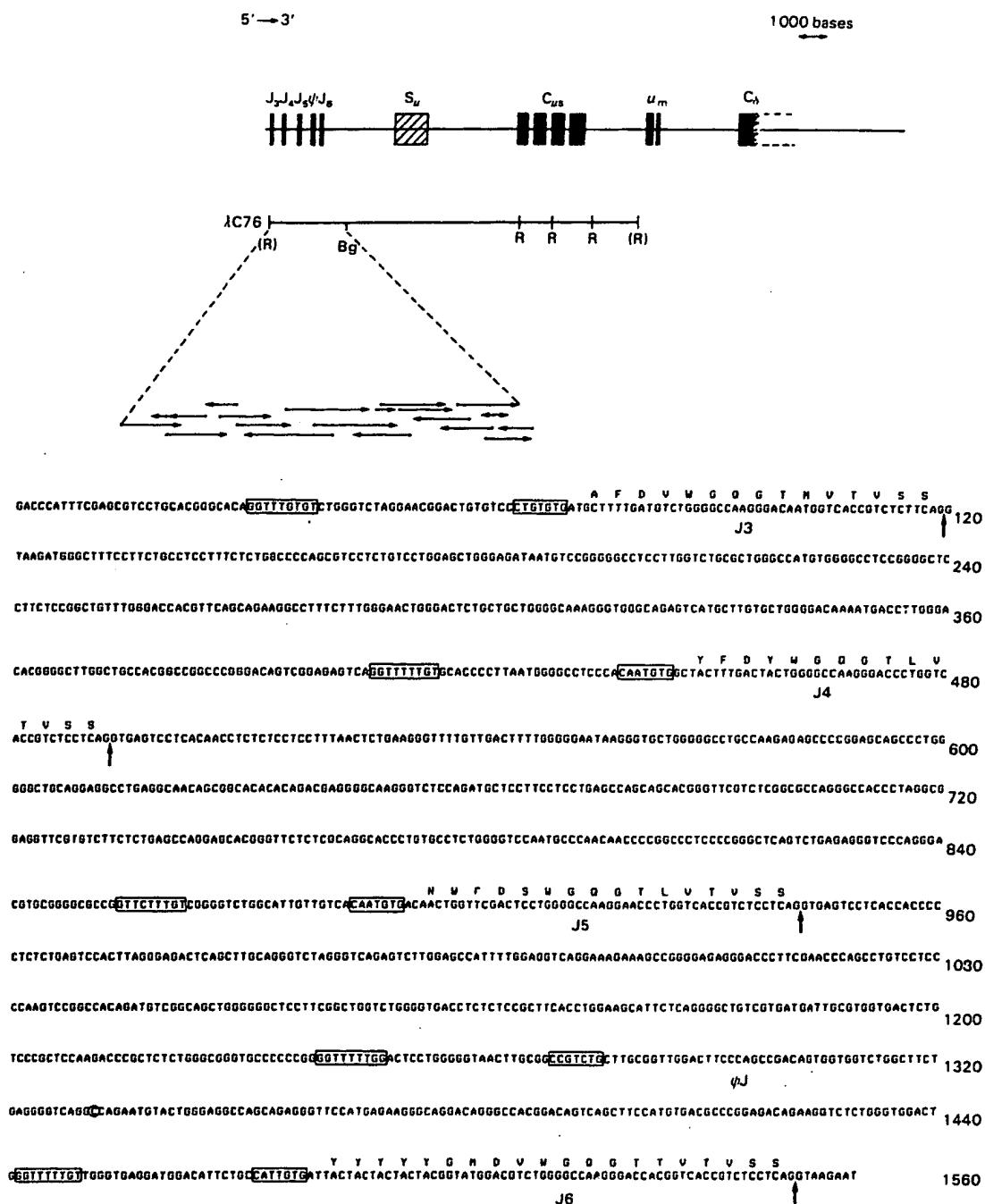


Fig. 5. Structural arrangement of the human genome encoding J_H -, μ - and δ -genes

Two recombinant phage clones carrying human foetal liver DNA were isolated by their hybridization to a mouse μ probe. Restriction mapping and nucleotide sequencing revealed the genomic structure shown in the top line (S = switch segment). The sequence of the coding regions of the J_H -segments are indicated and the RNA splicing sites are arrowed. The boxed sequences are signals thought to be important for the process of V-D-J joining.

Xenopus oocytes. We found that two randomly selected V_μ -genes were perfectly adequate templates for specific RNA synthesis in these systems. The transcription was found to start at a point approximately 30 bases downstream of a 'TATA' box (Corden *et al.*, 1980) and 20 bases upstream of the protein initiation codon. A third V_μ -pseudo-gene (which apparently

lacks appropriate signal sequence; see later) was unable to support specific RNA synthesis. We conclude from these experiments that sequences necessary for the transcription of mRNA are present on germ-line V_μ -genes. This shows that DNA rearrangement as such is not a pre-requisite for V-genes to be capable of transcription. Taken together with the observation

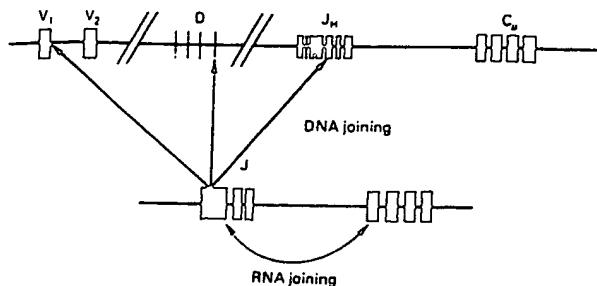


Fig. 6. Proposed events in the formation and transcription of the active heavy chain μ -gene

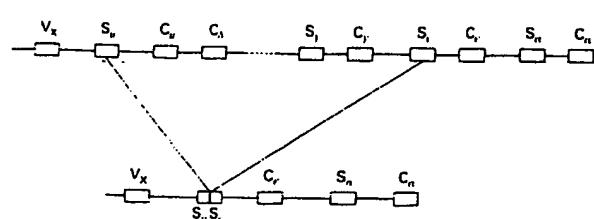


Fig. 7. Scheme for the process of switch recombination

that long stretches upstream of integrated mouse V-genes appear identical with germ-line counterparts argues that other factors than the immediate 5' sequence controls V-gene transcription.

The H-chain class switch: deletion of C_H -genes

After integration of the V_H /D/J segments, μ -chains appear in the cytoplasm of cells without L-chain. Subsequently L-chain integration occurs and the cells express surface IgM and can also express IgD. These cells carry the same V_H segment associated with the δ - or μ -chains. A further differentiation pathway, the H-chain class switch, takes a V_H -gene away from the C_H -gene and this V_H is transcribed with C_v , C_α or C_μ . Studies on the arrangement of C_H -genes in mouse myeloma cells expressing various classes of Ig showed that in plasma cells the switch is accompanied by deletion of genetic material between J_H and the newly expressed C_H -gene (Honjo & Kataoka, 1978; Cory & Adams, 1980; Coleclough *et al.*, 1980; Rabbitts *et al.*, 1980a). This deletion includes C_H -genes in the interim region. Detailed analysis of cloned C_H -genes from mouse myelomas revealed that this deletion was taking place between switch or S-segments located just upstream of each C_H except C_δ . The process of such a deletion is summarized in Fig. 7, which draws from our own work on mouse and human C_H -genes and that of various other laboratories (Davis *et al.*, 1980; Kataoka *et al.*, 1980; Sakano *et al.*, 1980). The switch recombination process seems to involve sequences within the S-segments which are located near each C_H -gene and which are homologous to each. Our studies of a mouse myeloma mutant IgG1 chain (Dunnick *et al.*, 1980) first identified short tandemly repeated regions apparently present in each C_H S-region which undergo rearrangements in the class switch. These segments were proposed to be mediators of S-S recombination, in a process such as unequal crossing over between sister chromatids (Fig. 8). This unequal crossing over would result in deletion of material and a switched genotype on one resulting chromosome (Rabbitts *et al.*, 1980b).

The involvement of these S-segments in recombination, of course, requires a sufficient homology between those of each C_H -gene. Such homology can be shown by hybridization and indeed such hybridization profiles reveal some interesting

features of the relative homologies of different S-segments. Fig. 9 shows the hybridization of a human S_μ probe with four γ -genes (putatively the active genes), the active ϵ -gene plus a ψ -gene ($\psi\epsilon_1$, which will be discussed later) and two α -genes (probably α_1 and α_2). Clearly all the C_H -genes analysed display some homology to the S_μ probe. By far the strongest homology is found in the α -genes and the active ϵ -gene. The $\psi\epsilon_1$ -gene does possess an S-sequence with significant homology to S_μ ; in fact, this homology is better than that of S_μ with the most homologous S_γ (i.e. $S_\gamma 3$). In general the S_γ hybridization was weak but none the less significant (Flanagan & Rabbitts, 1982b). This result is consistent with the similar result found in mouse and lends support to the theory that S_γ - S_γ recombination is by far the most likely after S_μ - S_μ switching (Marcu *et al.*, 1982).

Cotranscription of μ - and δ -genes as a means of C_H switching

We can conclude that switching of C_H -genes in plasma cells generally occurs by recombination between homologous S-segments and resulting in gene deletion. When we analysed the human δ -gene for an S-segment we found no evidence for such a sequence (Rabbitts *et al.*, 1981) so that an alternative mechanism was clearly operating in this case. Mapping of the μ - and δ -genes showed that the δ -gene was located about 5000 bases downstream of the μ -gene and in the same direction of transcription (Fig. 6). Roughly in the middle of the DNA separating these genes we located the coding regions for the membrane segment of μ (Rabbitts *et al.*, 1981) which are exactly analogous with those first described in mouse (Early *et al.*, 1980b). The proposal has been made that the membrane and secreted forms of μ result from differential RNA splicing routes which generate one or other type of mRNA (Early *et al.*, 1980b). The proximity of the δ - and μ -genes together with the absence of a detectable S-sequence argues that the μ - and δ -genes can be included in a single long transcript from which μ or δ mRNA is made containing the same V_H sequence. The inherent problem with such a scheme derives from the constraints on the RNA splicing mechanism, since in order to produce a VDJ δ mRNA, a set of RNA splicing sites has to be ignored (Milstein *et al.*, 1981).

Pseudo-genes in the human Ig locus

We have studied three ψ -Ig genes which include a ψV_μ -gene (Bentley & Rabbitts, 1980), a $\psi\gamma$ -gene (Krawinkel & Rabbitts, 1982), a $\psi\epsilon$ -gene (Flanagan & Rabbitts, 1982b) and a ψJ_H -segment (Flanagan & Rabbitts, 1982a). The structure of each ψ -gene precludes the possibility that they could function as active immunoglobulin genes and a number of interesting, different defects are found in the various genes. Each of the genes has a defective RNA splice site or at least a sequence at variance with the consensus splicing signal. Donor sites are affected in ψV_μ , J_H [two further ψJ_H with similar properties have also been identified (Ravetch *et al.*, 1981)] and $\psi\epsilon_1$, whilst an acceptor site is affected in $\psi\gamma$. The ψJ_H segments do not correspond to any known protein and several insertions/deletions place protein translation termination signals in phase. Similar insertion and deletion mutations were observed in the ψV_μ -gene which again potentially place in phase protein termination signals, even though we could identify the gene sequence as being closely related to the V_μ subgroup. The ψV_μ was also interestingly defective in signals for RNA transcription [i.e. no good match to the consensus TATA box sequence was evident and, indeed, this gene was not translatable *in vitro* (Bentley *et al.*, 1982)] and for V-J joining. Thus this ψ -gene is probably incapable of joining to a J-segment but, even if it were, transcription seems unlikely. In any event, transcription of this sequence would yield a pre-mRNA which would not be processed nor translatable into a full length protein. The most likely view of such ψ -genes is that they are relics of evolutionary drift which have gone beyond the limits of the constraint which may be imposed by function.

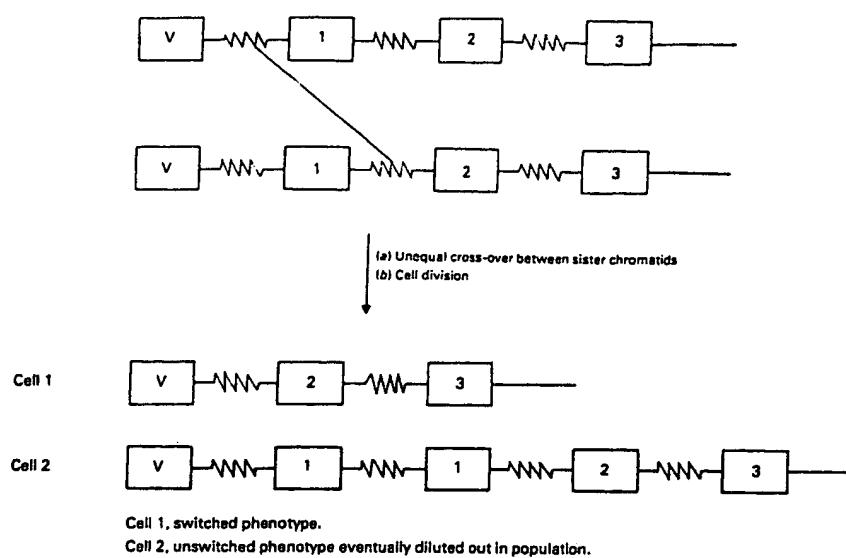


Fig. 8. Model for switch recombination by sister chromatid exchange

Genes 1 to 3 represent sequential C_H -genes. S-segments are represented as wavy lines, This is taken from Rabbitts *et al.* (1980b)

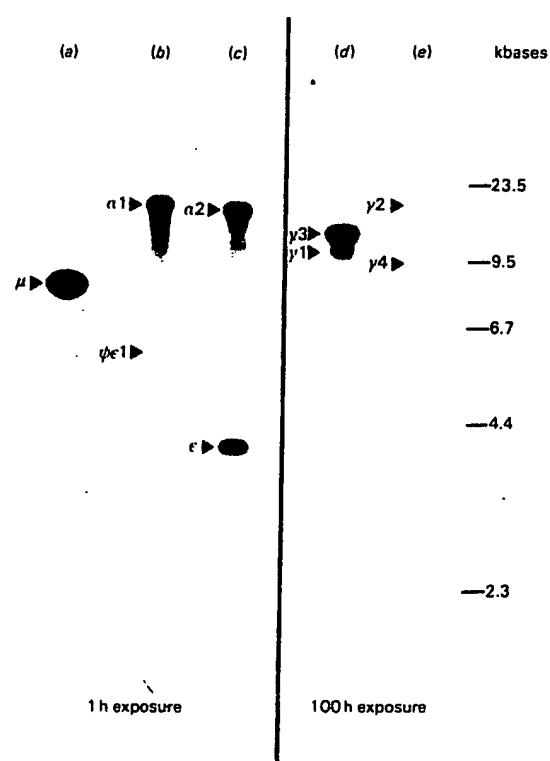


Fig. 9. Southern filter hybridization between S_{μ} - and S-segments from other C_H -genes

An S_{μ} probe was hybridized to restriction digests of cosmid clones carrying the various C_H -genes indicated in the Figure. After hybridization the filter was autoradiographed for the times indicated.

The γ_3 -gene, again, does not correspond to any known protein and apart from the known abnormality of a splice signal, may not possess an S-sequence (Takahashi *et al.*, 1982) thereby making it likely that it cannot normally be expressed. Finally the ψ_{ϵ_1} -gene is unusual in that it has lost the $C_{\epsilon}1$ and $C_{\epsilon}2$ domains completely and the first four residues of the $C_{\epsilon}3$ domain, which therefore includes the RNA splicing signal. Just in front of the site of deletion in this gene a sequence occurs which has a strong relationship with S-sequences. This type of deleted ψ -gene is reminiscent of the *in vitro* isolated IF2 mutant gene which we previously described (Dunnick *et al.*, 1980). The IF2 cell-line was discovered as an isoelectric focusing mutant, derived from the IgG1-producing mouse myeloma X63, which was subsequently shown to lack the complete $C_{\epsilon}1$ domain (Secher *et al.*, 1973). An analysis of the gene from IF2 showed that an extensive gene deletion had removed the $C_{\epsilon}1$ coding segment along with most of the $C_{\epsilon}1$ to hinge intervening sequence and a large part of the V_{ϵ} to $C_{\epsilon}1$ intervening sequence (Dunnick *et al.*, 1980). The mutant gene therefore has a structure which facilitated RNA splicing from the donor site of the V-region to the acceptor site of the hinge. A secretable H-chain was thus produced. The sequence of the gene showed that deletion had brought S-sequences very close to the hinge coding segment and thus the structures of ψ_{ϵ_1} and IF2 are very similar. The proximity of S-like sequences to the sites of deletion in these two genes is intriguing and suggests the involvement of these sequences in the formation of these mutant sequences. If this is true S-sequence must be important both in gene evolution and in derivations of mutant sequences in B-cells.

Arrangement of human heavy chain genes

The organization of the heavy chain locus in man is important as this region is expressed in a variety of malignant leukaemias. We wanted to be able to compare this region in normal and abnormal DNAs so a map of this gene region was required. We have used both V_H and C_H -region probes to analyse the heavy chain locus. Analysis of various human DNAs with a V_H^{III} probe indicated a complex picture of around 20 detect-

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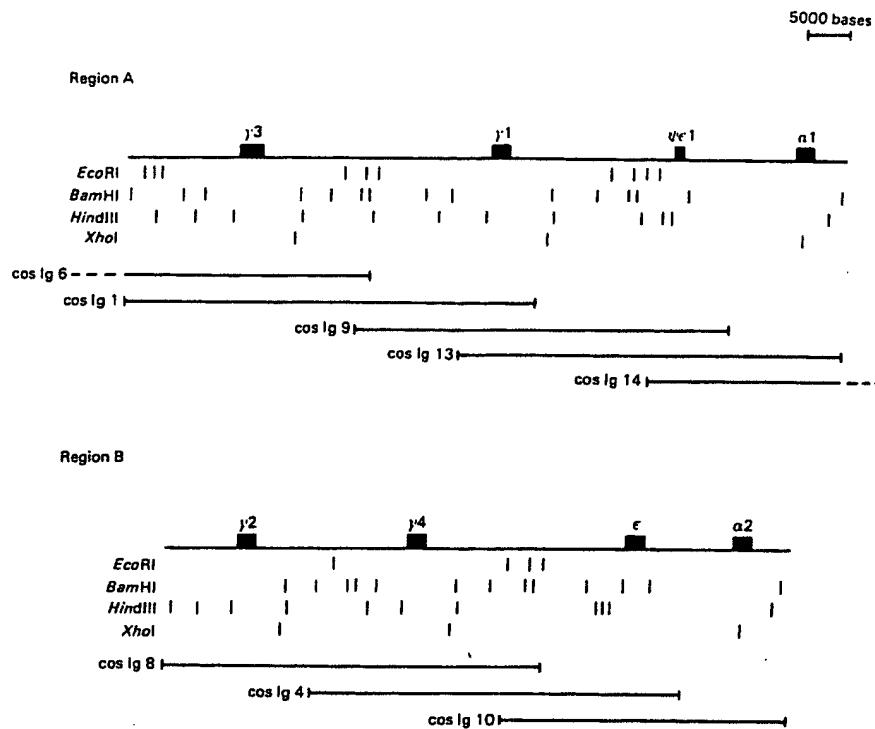


Fig. 10. Partial map of the human C_H -gene region

Cosmid clones were isolated by hybridization with γ and ϵ probes and restriction maps prepared. The broken lines at the ends of groups refer to the regions in which only a single clone has been mapped.

known signal, thereby ally the domains, which of the strong gene is such we cell-line d from subse- et al., hat an segment and a nick et which gion to is thus on had nt and r. The se two these this is and in able bands (Matthyssens & Rabbitts, 1980) and the analysis of this region is in its early stages. We studied the arrangement of V_H -segments in five randomly selected genomic clones and determined that the V -genes are separated from one another by quite a large distance; one clone contained two V_H -genes at a distance of 12500 bases whilst a second clone contained two V_H -genes about 15000 bases apart. The other clones analysed contained only one V -gene with the maximum size of genomic DNA, lacking a detectable V -gene, being about 16000 bases. Given an average spacing of 12000 bases and the existence of 100 V_H -genes, this implies that the V_H locus of chromosome 14 occupies at least 1200000 bases.

Recent studies of the human C_H -genes indicate that these genes also encompass a large region of chromosome 14. We have analysed C_H -genes by isolating cosmid clones from a human placental DNA library (prepared by F. Grosveld) and the structure of these clones revealed an interesting organization of genes. Fig. 10 shows the restriction maps of two non-overlapping groups of clones (each containing about 80000 bases of the genome) in which we have placed the relative positions of γ , ϵ and α -genes. The outstanding feature is that both groups of genes possess an order $\gamma-\gamma-\epsilon-\alpha$, implying that the evolution of this locus involved a vast duplication, probably including a segment with the arrangement $\gamma-\gamma-\epsilon-\alpha$ or $\gamma-\epsilon-\alpha$. This latter duplication was presumably followed by further duplication of γ -genes at each site. This interpretation is more compelling than the other possibility that the initial large duplication included $\gamma-\gamma-\epsilon-\alpha$, since comparison of restriction maps and sequences of the pairs of adjacent γ -genes indicates their close relationship. A complication to this general scheme is the existence of the $\psi\gamma$ -gene and to a lesser extent by the unusual

quadruplicated hinge segments found in the γ_3 -gene (Krawinkel & Rabbitts, 1982; Takahashi et al., 1982). Interestingly the first hinge of the γ_3 -gene is closely related to the $\psi\gamma$ hinge, implying an evolutionary relationship between γ_3 and $\psi\gamma_1$. The evolutionary origin of $\psi\gamma_1$ will not become clear until it is placed relative to the other γ -genes and even then the peculiarity of its sequence may preclude a clear conclusion regarding its origin. The study of the C_H -genes in other primates will be very interesting from the point of view of the nature of gene duplications and the origin of the ψ -genes.

Conclusions

The immunoglobulin locus of man is a highly fluid gene system which employs a variety of gene alteration events, in the maturation of the B-lymphocyte, resulting in expansion of diversity in the system. This system has evolved a series of signals which allow chromosomal rearrangement to occur in a specific way. However, it now seems that the very systems which contribute so elegantly to antibody activity may also result in the very occasional specific chromosomal translocations resulting in malignant leukaemias such as Burkitt lymphoma.

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Bentley, D. L., Farrell, P. J. & Rabbitts, T. H. (1982) *Nucleic Acids Res.* **10**, 1841-1856
 Bentley, D. L. & Rabbitts, T. H. (1980) *Nature (London)* **288**, 730-733

Bentley, D. L. & Rabbitts, T. H. (1981) *Cell* **24**, 613-633

Bernheim, A., Berger, R. & Lenoir, G. (1981) *Cancer Genet. Cytogenet.* **3**, 307-315

Coleclough, C., Cooper, D. & Perry, R. P. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1422-1426

Cooper, M. D., Kearney, J. F., Lydyard, P. M., Grossi, C. E. & Lawson, A. R. (1976) *Cold Spring Harbor Symp. Quant. Biol.* **41**, 139-191

Corden, J., Waslylyk, B., Buchwalder, A., Sassone-Corsi, P., Kedinger, C. & Chamson, P. (1980) *Science* **209**, 1406-1414

Cory, S. & Adams, J. M. (1980) *Cell* **19**, 37-51

Croce, C. M., Shander, M., Martinis, J., Cicurel, L., D'Amona, G. G., Dolby, T. W. & Koprowski, H. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3416-3419

Davis, M. M., Calame, K., Early, P. W., Livant, D. L., Joho, R., Weissman, I. L. & Hood, L. (1980) *Nature (London)* **283**, 733-739

Dunnick, W., Rabbitts, T. H. & Milstein, C. (1980) *Nature (London)* **286**, 669-675

Early, P., Huang, H., Davis, M., Calame, K. & Hood, L. (1980a) **19**, 981-998

Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R. & Hood, L. (1980b) *Cell* **20**, 313-319

Ellison, J. & Hood, L. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1984-1988

Erikson, J., Martinis, J. & Croce, C. M. (1981) *Nature (London)* **294**, 173-175

Flanagan, J. G. & Rabbitts, T. H. (1982a) *EMBO J.* **1**, 655-660

Flanagan, J. G. & Rabbitts, T. H. (1982b) *Nature (London)* **300**, 709-713

Hobart, M. J., Rabbitts, T. H., Goodfellow, P. N., Solomon, E., Chambers, S., Spurr, N. & Povey, S. (1981) *Ann. Hum. Genet.* **45**, 331-335

Honjo, T. & Kataoka, T. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2140-2144

Hozumi, N. & Tonegawa, S. (1976) *Proc. Natl. Sci. U.S.A.* **73**, 3628-3632

Karpas, A., Hayhoe, F. G. J., Greenberger, J. S., Barker, C. R., Cawley, J. C., Lowenthal, R. M. & Moloney, W. C. (1977) *Leukaemia Res.* **1**, 35-49

Kataoka, T., Kawakami, T., Takahashi, N. & Honjo, T. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 919-923

Kirsch, I. R., Morton, C. C., Nakahara, K. & Leder, P. (1982) *Science* **216**, 301-303

Klein, E., Klein, G., Nadkarni, J. S., Nadkarni, J. J., Wigzell, H. & Clifford, P. (1968) *Cancer Res.* **28**, 1300-1310

Klein, G. (1981) *Nature (London)* **294**, 313-318

Knapp, W., Bolhuis, R. L. H., Rádl, J. & Hijmans, W. (1973) *J. Immunol.* **111**, 1295-1298

Krawinkel, U. & Rabbitts, T. H. (1982) *EMBO J.* **1**, 403-407

Krumlauf, R., Jeanpierre, M. & Young, B. D. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2971-2975

Kurosawa, Y. & Tonegawa, S. (1982) *J. Exp. Med.* **155**, 201-218

McBride, O. W., Hieter, P. A., Hollis, G. F., Swan, D., Otey, M. C. & Leder, P. (1982) *J. Exp. Med.* **155**, 1480-1490

Malcolm, S., Barton, P., Murphy, C., Ferguson-Smith, M. A., Bentley, D. L. & Rabbitts, T. H. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4957-4961

Marcu, K. B., Lang, R. B., Stanton, L. W. & Harris, L. J. (1982) *Nature (London)* **298**, 87-89

Mather, E. & Perry, R. P. (1981) *Nucleic Acids Res.* **9**, 6855-6867

Matthyssens, G. & Rabbitts, T. H. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6561-6565

Max, E. E., Seidman, J. G. & Leder, P. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3450-3454

Milstein, C., Burrone, O. R., Dunnick, W., Milstein, C. P. & Rabbitts, T. H. (1981) in *Mechanisms of Lymphocyte Activation* (Resch & Kirchner, eds.), pp. 63-78, Elsevier, North-Holland, Amsterdam

Minowada, J., Ohnuma, T. & Moore, G. E. (1972) *J. Nat. Cancer Inst.* **49**, 891-895

Pernis, B., Forni, L. & Luzzatti, A. L. (1976) *Cold Spring Harbor Symp. Quant. Biol.* **41**, 175-183

Rabbitts, T. H. (1978) *Nature (London)* **275**, 291-296

Rabbitts, T. H. & Forster, A. (1978) *Cell* **13**, 319-327

Rabbitts, T. H., Forster, A., Dunnick, W. & Bentley, D. L. (1980a) *Nature (London)* **283**, 351-356

Rabbitts, T. H., Hamlyn, P. H., Matthyssens, G. & Roe, B. A. (1980b) *Can. J. Biochem.* **58**, 176-187

Rabbitts, T. H., Forster, A. & Milstein, C. P. (1981) *Nucleic Acids Res.* **9**, 4509-4524

Ravetch, J. V., Siebenlist, U., Korsmeyer, S., Waldmann, T. & Leder, P. (1981) *Cell* **27**, 583-591

Rowley, J. D. (1982) *Science* **216**, 749-751

Sakano, H., Hüppi, K., Heinrich, G. & Tonegawa, S. (1979) *Nature (London)* **280**, 288-294

Sakano, H., Maki, R., Kurosawa, Y., Roeder, W. & Tonegawa, S. (1980) *Nature (London)* **286**, 676-680

Secher, D. S., Cotton, R. G. H. & Milstein, C. (1973) *FEBS Lett.* **37**, 311-316

Siebenlist, U., Ravetch, J. V., Korsmeyer, S., Waldmann, T. & Leder, P. (1981) *Nature (London)* **294**, 631-635

Sledge, C., Fair, D. S., Black, B., Krueger, R. G. & Lood, L. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 923-927

Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503-517

Takahashi, N., Ueda, S., Obata, M., Nikaido, T., Nakai, S. & Honjo, T. (1982) *Cell* **29**, 671-679

Wang, A. C., Wilson, S. K., Hopper, J. E., Fudenberg, H. H. & Nisonoff, A. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **66**, 337-343

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Sequence of the Human Immunoglobulin Diversity (D) Segment Locus: A Systematic Analysis Provides No Evidence for the Use of DIR Segments, Inverted D Segments, "Minor" D Segments or D-D Recombination

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We have determined the complete nucleotide sequence of the human immunoglobulin D segment locus on chromosome 14q32.3 and identified a total of 27 D segments, of which nine are new. Comparison with a database of rearranged heavy chain sequences indicates that the human antibody repertoire is created by VDJ recombination involving 25 of these 27 D segments, extensive processing at the V-D and D-J junctions and use of multiple reading frames. We could find no evidence for the proposed use of DIR segments, inverted D segments, "minor" D segments or D-D recombination. Conventional VDJ recombination, which obeys the 12/23 rule, is therefore sufficient to explain the wealth of lengths and sequences for the third hypervariable loop of human heavy chains.

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Keywords: diversity; D segment; germline; immunoglobulin; recombination

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Introduction

Antigen binding by antibodies is mediated by six polypeptide loops: three from the heavy chain variable domain (H1, H2 and H3) and three from the light chain variable domain (L1, L2 and L3). Although all six loops are variable in sequence and length, the H3 loop, which is located at the centre of the antigen binding site and makes more contacts with antigen than any other loop (Wilson & Stanfield, 1994), is by far the most diverse (Kabat *et al.*, 1991). Diversity in this region is generated by VDJ recombination of three sets of germline gene segments: variable (V_H), diversity (D) and joining (J_H) (Tonegawa, 1983). Joining is directed by specific recombination signal sequences (RSS), which flank the coding sequence of every germline gene segment. Each RSS consists of a conserved palindromic heptamer sequence separated from a conserved nonamer sequence by a 12 or 23 bp spacer. Only segments with differently sized

spacers may be joined according to the "12/23 rule". Nucleotides are often removed at the site of recombination and a variable number of non-template N-nucleotides added by terminal deoxynucleotidyl transferase (TdT; Alt & Baltimore, 1982; Komori *et al.*, 1993). Thus, the sequences of the D segments and the way in which they join to the V_H and J_H segments are major factors in determining antigen specificity.

The human germline V_H and J_H segments have now been completely mapped and sequenced. There are approximately 51 functional V_H segments, depending on the haplotype (Tomlinson *et al.*, 1992; Cook & Tomlinson, 1995), and six functional J_H segments (Ravetch *et al.*, 1981). In contrast, the total number of functional D segments and their sequences are unknown. Indeed, of the estimated 30 segments on chromosome 14 (the "major" D segment locus), only 18 have been sequenced (Ravetch *et al.*, 1981; Siebenlist *et al.*, 1981; Buluwela *et al.*, 1988; Ichihara *et al.*, 1988). In addition, it is not completely understood how these D segments are incorporated into the H3 region. Conventional VDJ recombination involves the joining of the 3' RSS of the D segment with the 5' RSS of the J_H segment, followed by the joining of the 5' RSS of the D segment with the 3' RSS of the V_H

Abbreviations used: V_H , heavy chain variable gene segment; D, heavy chain diversity gene segment; J_H , heavy chain joining gene segment; RSS, recombination signal sequence; TdT, terminal deoxynucleotidyl transferase.

segment. However, it has also been suggested that D segments may use the 5' instead of the 3' RSS to recombine to a J₁₁ segment (in this case, the D segment would be inverted; Gellert, 1992; Tuaillon *et al.*, 1995) and that D segments may recombine with each other (D-D recombination, flouting the 12/23 rule; Sanz, 1991; Yamada *et al.*, 1991; Brezinschek *et al.*, 1995). It has also been suggested that additional elements may be incorporated into the H3 loop. These include the DIR segments, longer sequences interspersed amongst the functional D segments that are flanked by multiple 12 and 23 bp spacer RSS (Ichihara *et al.*, 1988; Sanz *et al.*, 1994) and the "minor" D segments (a cluster of D segments on chromosome 15; Matsuda *et al.*, 1988, 1990; Nagaoka *et al.*, 1994; Tomlinson *et al.*, 1994). Since the evidence for these unconventional mechanisms is based on short sequence homologies with many mismatches and insertions/deletions (Sanz, 1991; Yamada *et al.*, 1991) it is difficult to verify their existence without a complete knowledge of the sequences of all the germ-line D segments.

To establish the precise mechanism involved in VDJ recombination, we therefore determined the complete nucleotide sequence of the major D segment locus on chromosome 14q32.3. We then used a systematic and quantitative method of scoring sequence homologies with somatically rearranged heavy chains to identify all the germline D segments (and any other sequences) that contribute to H3 diversity.

Results

Complete nucleotide sequence of the D segment locus

A shotgun sequencing strategy (Wilson *et al.*, 1994) was used to sequence three cosmids (COS23, COS21 and COS24) that form a contig covering the entire D segment locus on chromosome 14q32.3 (Buluwela *et al.*, 1988). The nucleotide sequence of 92,588 bp (EMBL data library accession number X97051) was completely determined to high accuracy from a *Hind*III restriction site 13 kb upstream of the V_{H6} segment to the beginning of the μ switch region 2.7 kb downstream of the J_H segments (Figure 1). The distance from V_{H6} to J_H1 is therefore 74 kb.

There are 27 D segments between V_H 6 and the J_{μ} segments

In all, 27 conventional D segments were identified that have a short potential coding sequence (11 to 37 bp) flanked by two 12 bp spacer RSS (Figure 2). These can be grouped into seven families based on sequence homology. Six of these families (D_N , $D_{I,R}$, $D_{X,I}$, D_A , D_K and D_N ; Ichihara *et al.*, 1988) have at least four members, whilst the seventh is the unique D_{HQ52} segment. Within each family, the RSS are relatively conserved (consistent with recognition by the recombinase), whilst the coding sequences are more divergent (consistent with providing diversity for the H3 loop). In addition, one DIR segment was found immediately

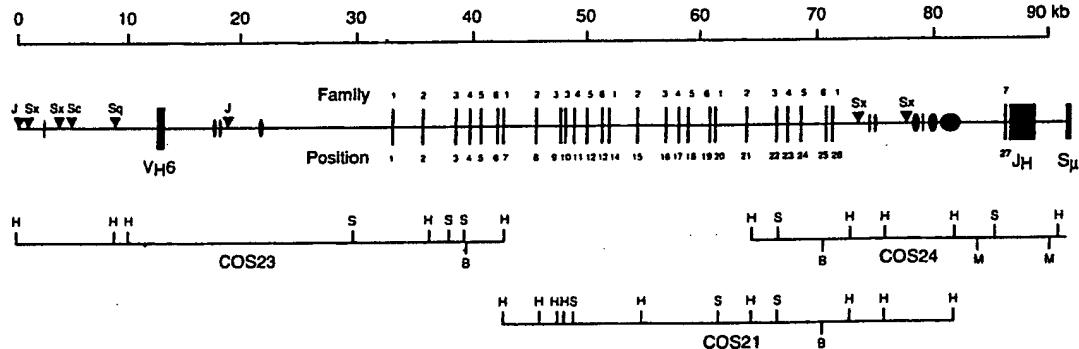


Figure 1. A scale map of the human immunoglobulin D segment locus based on the nucleotide sequences of the three cosmid clones, COS23, COS21 and COS24 (indicated below the map with the following restriction sites; H, *Hind*III; S, *Sfi*I; B, *Bss*HII; M, *Mlu*I). COS23 and COS21 have previously been shown to juxtapose (Buluwela & Rabbitts, 1988). D segments are represented by thin vertical lines, *Alu* elements by triangles and L1 elements by ovoids (the width of which indicates the approximate size of the element). The *Alu* sub-families are shown above each element. Where definite sub-family allocations for L1 elements could be made, all were to pre-primate radiation, mammalian-wide families. The V_H 6 segment, the J_H segments and the S_H region are shown as black rectangles. D segments are named according to the convention originally established for the human V_H segments (Shin *et al.*, 1991; Cook *et al.*, 1994). This introduces a two-number system, where the first number indicates the family (shown above each segment in the Figure) and the second number indicates the relative position of the segment in the locus from V_H to J_H (shown below each segment in the Figure). The new family assignments are therefore as follows: $D_{\alpha 1}$ becomes D1, $D_{\alpha 1R}$ becomes D2, $D_{\alpha 1P}$ becomes D3; $D_{\alpha 1}$ becomes D4, $D_{\alpha 2}$ becomes D5, $D_{\alpha 3}$ becomes D6 and D_{HQ52} becomes D7-27. The new names for all previously sequenced D segments are given in Figure 2.

New Name
DI-1
DI-7
DI-14
DI-20
DI-26
D2-2
D2-8
D2-15
D2-21
D3-3
D3-9
D3-10
D3-16
D3-22
D4-4
D4-11
D4-17
D4-23
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D6-6
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D7-27

Figure 2. groups with entity). For each prevent or established sequence ²Siebenlist et al. later renamed substitutions, second under respectively; underlined previously published COS21) (B1-D3-10 and I same as D5-

upstream of the D segment 14 have no naming consequences for the human V_H genes, since it indicates its family membership (see the legend). No evidence for a functional repertoire, s

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New Name	Old Name	Nonamer	12 bp Spacer	Heptamer	D Segment Coding Sequence	Heptamer	12 bp Spacer	Nonamer
D1-1		AGATTCGGA	ACAGGGCGGAGT	CACGCG	GTATACATGGAAACGAC		CACCGTG	AGAAAAACTGTG
D1-7	D _M ¹	G.....	A.....T.....T.....	T.....G.....TC.....G
D1-14	D _M ²	G.....	A.....T.....C.....C.....	T.....C.....T.....G.....AC.....A.....
D1-31		G.....	A.....T.....	T.....
D1-26		G.....T.....G.....G.....T.....TAC	T.....G.....A.....
D2-1	D _M ² , D _M ³	GGTTTTGT	GGGGGCTCTGT	CACGCG	GGGGATTTGAGTGTAGTACGGCTGTAT&CC	CACAGTG	ACACAGCTCCAT	TCCAAAAGC
D2-8	D _M ¹ (D _M ²)C.....A.....GGTGA.....
D2-15	D _M ²G.....GGT.....CT.....A.....
D2-21	(D _M ²)C.....G.....G.....A.....T.....A.....T.....
D3-3	D _M ⁴ (D23/7 ⁴)	GGTTTGGG	GGAGGGCTCTGT	CACGCG	GTATACGATT---TTGGAGTGGTAT---TATACC	CACAGTG	TCACAGAGTCGA	TCAAAAAC
D3-9	D _M ¹ , D21/0, 5 ⁴AAA.....T.....C.....A.....
D3-10	D _M ¹ (D21/7 ⁴)T.....G.....G.....C.G.GA.....A.....
D3-16	(D21/10 ⁴)AAT.....ACG.....G.GA.....CGT.....CA.....CG.....G.....
D3-22	D21/9 ⁴AAT.....G.....A.....T.....CTA.....T.....
D4-4	D _M ⁴	GCTTTTGT	GAAGGGCTCC	TACTGTG	TGACTAC---AGTAAC	CACAGTG	ATGAACCCAGCA	GCAAAAC
D4-11	D _M ¹C.....G.....T.....TG.....
D4-17	CC.....A.....T.....
D4-23	C.....G.....GGTG.....C.....A.....
D5-5	D _M ¹	GGTTATGT	CAGGGGGTGTCA	GACTGTG	GTGGATACTGTA---TGGTAC	CACAGTG	GTGGCTGCCATA	GCAGCAACC
D5-12	D _M ¹C.A.....T.....TGGCTAC.A.....C.....
D5-18	
D5-24	C.....G.C.....A.....G.TG.....CAA.....C.....
D6-6	(D _M ¹)	AGTTCTGA	AGGTGCTCTGT	CACGCG	GAGTATAGCA---GCTGTC	CACAGTG	ACACTCGCCAGG	CCAGAAAC
D6-13	D _M ¹G.....GCA.....G.A.....A.....CA.....
D6-19	C.....A.....G.....GTG.....G.A.....
D6-25	C.....C.....G.....GGC.A.....A.....G.G.....A.....
D7-27	(D _{HQ52} ⁵)	GGTTTGGC	TGAGCTGAGAAC	CACTGTG	CTAATCTGGGG	CACAGTG	ATTGGCAGCTCT	ACAAAAACC

Figure 2. Nucleotide sequences of the human D segments on chromosome 14q32.3. Sequences are aligned in family groups with dots to indicate nucleotide homology and dashes to indicate pad characters (inserted to maximise identity). For each D segment the RSS heptamers and nonamers are boxed. Nucleotides that would be expected to prevent or severely restrict recombination (Gellert, 1992; Akamatsu *et al.*, 1994) are circled. Names of previously published sequences are given next to their corresponding D segments. References are ¹Ichihara *et al.* (1988); ²Siebenlist *et al.* (1981); ³ Zong *et al.* (1988); ⁴ Buluwela *et al.* (1988); ⁵ Ravetch *et al.* (1981). Note that D₂, D₃ and D₄ were later renamed D_{LR2}, D_{LR3} and D_{LR4} (Ichihara *et al.*, 1988). Sequence names in parentheses have a number of nucleotide substitutions (underlined positions) compared to our sequences. These are: D₁ (allele of D2-2) with A → G at the second underlined position; D_{1-b} (allele of D2-2) with A → T and A → G at the first and second underlined positions, respectively; D₁ (allele of D2-8) with AA → GG, D₃ (allele of D2-21) with T → C and C → T at the first and second underlined positions, respectively; D_{N1} (allele of D6-6) with A → C; and D_{HQ52} (allele of D7-27) with G → T. The previously published sequences of D23/7 (from cosmid COS23), D21/7 (from cosmid COS21), D21/10 (from cosmid COS21) (Buluwela *et al.*, 1988) are incorrect and are therefore superseded by the sequences of D3-3, D3-10 and D3-16 shown here. Note that the coding sequence of D4-4 is the same as D4-11 and that of D5-5 is the same as D5-18.

upstream of each of the five D_M segments. As all the D segments in the major locus on chromosome 14 have now been identified, we propose a new naming convention (similar to the one used for the human V_H segments) that for each D segment indicates its family and its relative position in the locus (see the legend to Figure 1 and Figure 2). (DIR segments were omitted from this scheme as there is no evidence that they contribute to the functional repertoire, see below.)

Comparison of our and previously published D segment sequences indicates limited allelic polymorphism (see Figure 2). The sequences of all human germline D segments can be obtained from the V BASE directory of human immunoglobulin genes (available on the World Wide Web: <http://www.mrc-cpe.cam.ac.uk/imt-doc/vbase-home-page.html> or on disk from I.M.T.).

www.mrc-cpe.cam.ac.uk/imt-doc/vbase-home-page.html or on disk from I.M.T.).

Organisation and evolution of the D segment locus

The complete nucleotide sequence of the D segment locus on chromosome 14 confirms that the major D segments are arranged in four 9.5 kb tandem repeat units, with the same order of D segment families in each cluster (Figure 3a; Siebenlist *et al.*, 1981; Buluwela *et al.*, 1988; Ichihara *et al.*, 1988; Zong *et al.*, 1988). The high degree of sequence homology is illustrated by a two-dimensional dot matrix plot of this region (Figure 3b). In addition to the four 9.5 kb repeats there is a 2.8 kb

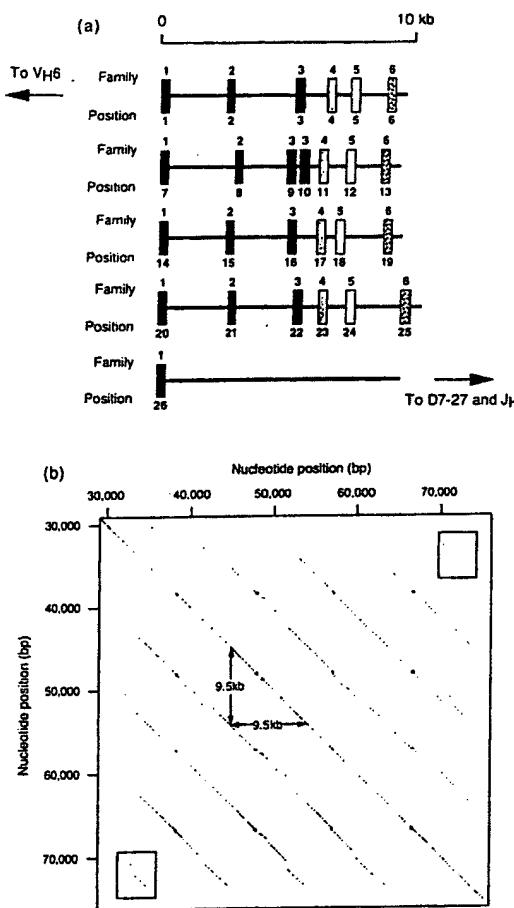


Figure 3. Repeat structure of the central D segment cluster. **a**, A representation of the central D segment cluster containing 26 segments (drawn to scale). The six families are represented by different shading. The family is shown above and the position is shown below each segment. **b**, Two-dimensional dot plot created with the program Dotter (Sonnhammer & Durbin, 1995) from nucleotides 29,000 to 76,000 (as shown in Figure 1) which encompasses the central D segment cluster. The shortest distance between repeats (indicated by arrows) is, on average, 9.5 kb. In addition to four 9.5 kb repeats there is a region of homology of 2.8 kb shown boxed at the bottom left and top right of the diagram. Breaks in the continuity of the diagonals indicate small-scale insertions/deletions from one repeat unit to the next.

region homologous to one end of a full repeat. This contains an extra D1 segment (boxed in Figure 3b).

Although the locus is G + C-rich (54.9%) and *Alu* repeat elements tend to concentrate in G + C-rich regions of the genome (Korenberg & Rykowski, 1988), the density of *Alu* repeats (0.05/kb) is lower than the lowest previously reported for a large human sequence (0.1/kb; Legouis *et al.*, 1991; Whitfield *et al.*, 1995). Indeed, the central D seg-

ment cluster (D1-1 to D1-26) lacks *Alu* and L1 elements altogether (Figure 1). This suggests that the primordial locus lacked *Alu*/L1 elements and that the flanking *Alu*/L1 elements were spread apart by repeated duplication of the D segments. If true, this indicates that the expansion of the central D segment locus occurred within the last 33 million years (when the youngest *Alu* elements, *Alu*-S, were dispersed throughout the genome; Zietkiewicz *et al.*, 1994). This is consistent with the different organisation and family classification of the central D segment cluster in mice (Ichihara *et al.*, 1989; Feeney & Riblet, 1993). The only D segment in humans (D7-27/D_{H052}, Figure 1) related to a mouse D segment (D₀₅₂, Ravetch *et al.*, 1981) is located in a similar position immediately upstream of the J_H segments, indicating that this segment was present prior to the divergence of the human and mouse lineages.

Alignment of rearranged heavy chain H3 sequences

To assign somatically rearranged sequences to their germline V, D and J counterparts, we used the BLAST algorithm (Altschul *et al.*, 1990). BLAST does not insert gaps to maximise homology between query and target sequences and produces a numerical score for the best alignment. The BLAST algorithm was incorporated into a UNIX-based sequence alignment package called Germline Query (GQ), which takes the sequence of a rearranged antibody gene and systematically searches a directory of germline gene segments (V BASE) for the best matches to V and J segments (Figure 4). With heavy chain genes, GQ masks the region of the query sequence that aligns to V_H and J_H and then searches for D segments in the intervening H3 sequence.

In all, 893 different heavy chain sequences with identifiable V_H and J_H sequences were extracted from a large database of rearranged human heavy chain sequences (Tomlinson *et al.*, 1996) and used as the source of query sequences for GQ. We also compiled a database of target D sequences, corresponding to the nucleotide sequences (both strands) of the 27 germline D segments and their alleles (see Figure 2), the five D segment sequences from chromosome 15 (Matsuda *et al.*, 1988, 1990; Nagaoka *et al.*, 1994; Tomlinson *et al.*, 1994) and the five DIR segments (from this study).

In previous studies, the criteria for assigning H3 sequences to their germline D segments appear to have been far too lenient. For example, others (Mortari *et al.*, 1993; Brezinschek *et al.*, 1995) have taken five nucleotides of identity or six nucleotides with one mismatch as their minimum cut-off for identifying a germline sequence. In the complete D segment database of 3978 bp (see above) even a random sequence for H3 is likely to find a D segment match according to these criteria (there are only 1024 possible sequence permutations of five

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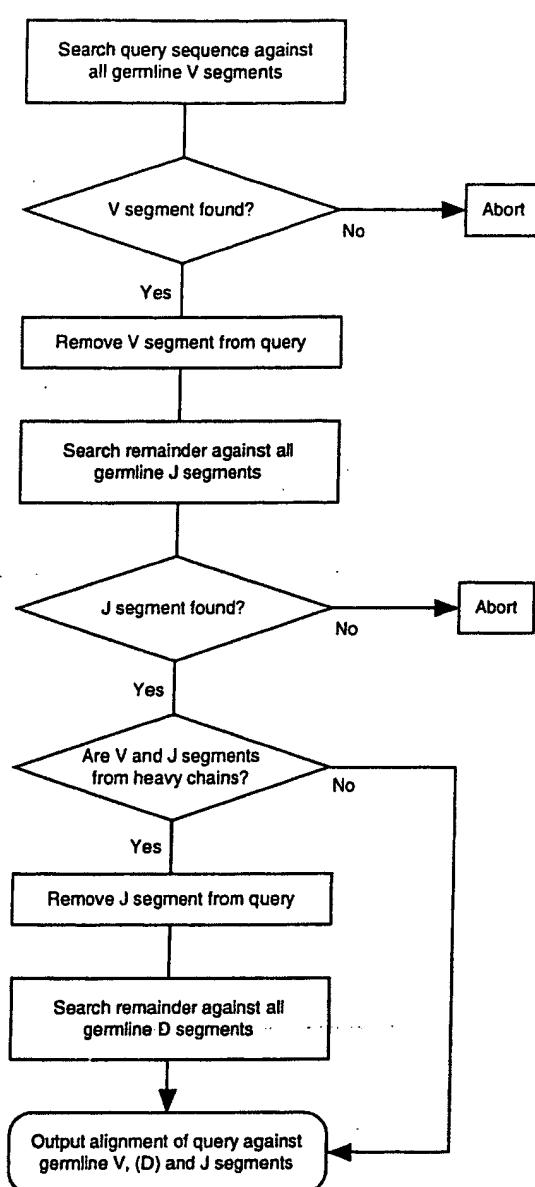


Figure 4. Flow-chart for the GQ alignment software.

nucleotides). Thus, many of these previous alignments are probably incorrect.

As a control for D segment assignment, we therefore generated 28,000 "mock" heavy chains with random H3 sequences of varying lengths sandwiched between genuine V_H and J_H sequences (H3 length ranged from five to 70 nucleotides in intervals of five nucleotides, with 2000 sequences of each length). The scores produced by aligning these sequences to the germline D segment database provided a "threshold of confidence" for alignment of real heavy chain H3 sequences. Our criteria were stringent: if the

score for an H3 alignment rose above the 99th percentile score produced by mock sequences of the same length the alignment was considered plausible. Even with this high threshold of confidence we would expect 1 in 100 alignments to be due to chance and therefore be incorrect. Using this approach, we found that at least ten consecutive nucleotides of identity are generally required to confidently assign a D segment, although the precise threshold depends on the H3 length. To validate our criteria we compared the 893 somatically rearranged heavy chain sequences with the germline D segment database. Reassuringly, the scores for the majority of H3 sequences aligned to D segments from the seven families rise above the 99% threshold (Figure 5a).

No evidence for the use of DIR segments, inverted D segments, "minor" D segments or D-D recombination

The contribution of DIR segments to the heavy chain repertoire was determined by comparing the scores of somatic H3 sequences assigned to DIR segments with our threshold of confidence (Figure 5b). Only six H3 alignments to DIR segments rise above the 99% confidence level, providing little evidence for the use of DIR segments. The DIR segments were therefore removed from the germline D segment database for subsequent alignments.

We then checked for evidence of D segments that rearrange by inversion. Scores produced by alignment to the positive (defined as the one that runs 5' to 3' from V_H to J_H) and negative DNA strands (defined as the one that runs 5' to 3' from J_H to V_H) are shown in Figure 5c and d, respectively. Only seven alignments to the negative strand rise above the 99% confidence threshold, in marked contrast to positive strand alignments, providing little evidence for inversion of D segments during recombination. Subsequent H3 alignments were therefore performed using only the positive strand.

Next, we looked at the contribution of the minor D segments on chromosome 15. The scores produced by alignment of H3 sequences to D segments on chromosomes 14 and 15 are shown in Figure 5e and f, respectively. Only six alignments for the chromosome 15 segments rise above the 99% confidence level. Given that the V_H sequences associated with these H3 alignments are highly mutated (>7.9% nucleotide differences), these probably represent mutated H3 sequences derived from major D segments. Hence, there is little evidence for use of minor D segments. The minor D segments were therefore removed from the germline D segment database for subsequent alignments.

We then searched for evidence of multiple D segment alignments. Any H3 sequence that remained after confident assignment of a first D segment (Figure 5g) was again assigned to the

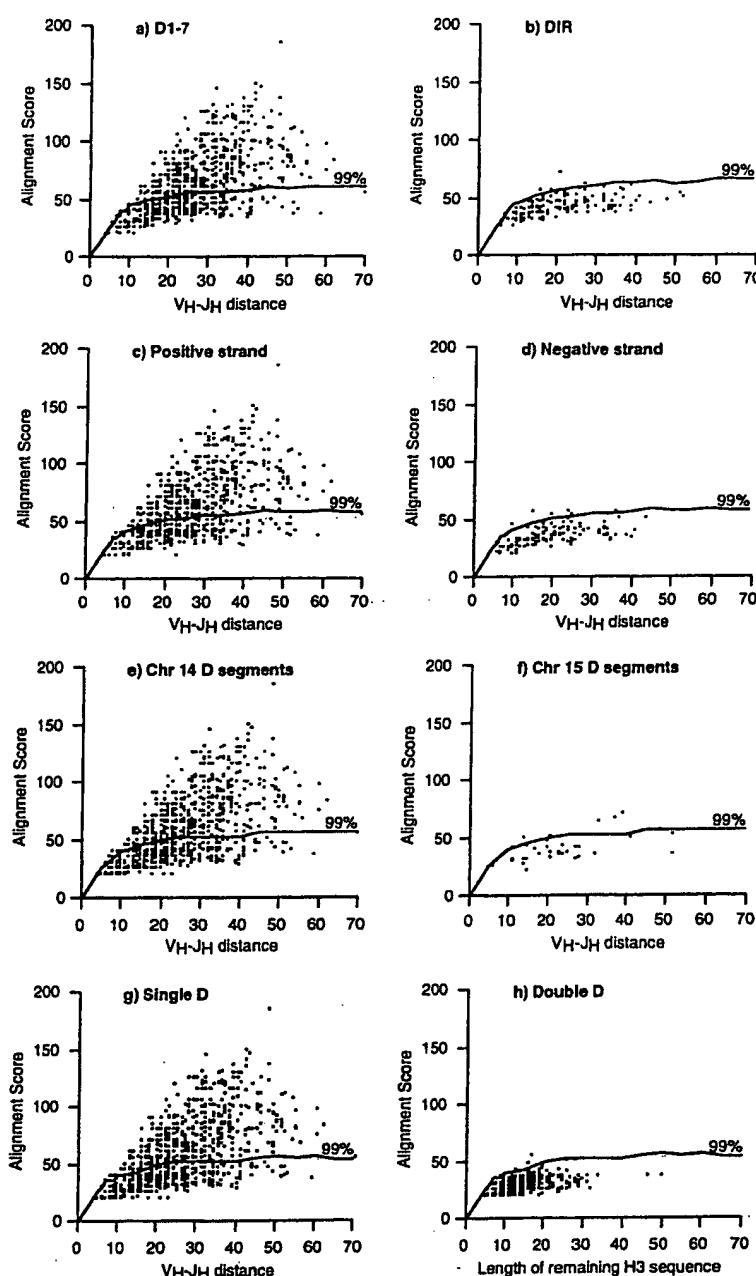


Figure 5. Systematic assignment of somatically rearranged H3 sequences to germline D segments. The scores produced by the best alignment found by GQ when 893 H3 sequences were searched against the germline D segment database are plotted as dots according to the length of the nucleotide sequence used for D segment alignment (the region between the end of V_H homology and the beginning of J_H homology: the V_H-J_H distance). Black lines show the 99th percentile score produced by random sequences aligned to the same category of D segments (see the text). For example, the black line in b corresponds to the 99th percentile of random H3 sequences assigned to DIR segments. Note that where two or more alignments have the same score and V_H-J_H distance, a single dot is shown. a, Alignments to D1-7 family members when H3 sequences were screened against a germline D segment database containing both strands of the 27 D segments on chromosome 14 (including their alleles), the five D segment sequences from chromosome 15 and the five DIR segments (from this study). Out of 739 alignments, 399 are above the 99% confidence level (the rest of the 893 rearranged H3 sequences align to DIR segments, see b, below). b, Alignments to DIR segments when H3 sequences were screened against the same germline D segment database used in a. Only six out of 154 alignments are above the 99% confidence level (the rest of the 893 rearranged H3 sequences align to D1-7 segments, see a, above). c, Alignments to the positive DNA strand (defined as the one that runs 5' to 3' from V_H to J_H) when H3 sequences were screened against a germline D segment database containing both strands of the 27 D segments on chromosome 14 (including their alleles) and the five D segment sequences from chromosome 15. Out of 860 alignments, 454 are above the 99% confidence level (the rest of the 893 rearranged H3 sequences align to minor D segments, see f, below). f, Alignments to minor D segments from chromosome 15 when H3 sequences were screened against the same germline D segment database used in e. Six out of 33 alignments are above the 99% confidence level (the rest of the 893 rearranged H3 sequences align to major D segments, see e, above). g, Alignments to a first D segment when H3 sequences were screened against a germline D segment database containing the 5' to 3' (V_H to J_H) strand of the 27 D segments on chromosome 14 (including their alleles). Out of 893 alignments, 451 are above the 99% confidence level. h, Alignments to a second D segment when the remaining H3 regions 5' and 3' of the first D segment alignment (see above) were screened against the same germline D segment database used in g. Only four out of 821 alignments are above the 99% confidence level.

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Table 1. Nucleotide composition of H3 sequences

	%"G	%"C	%"A	%"T	Average nucleotide length
Total N-nucleotide addition	32.6	28.8	19.1	19.5	13.6
V _H end N-nucleotide addition	32.0	28.9	18.6	20.5	7.3
J _H end N-nucleotide addition	33.3	28.7	19.5	18.4	6.3
No D segment in H3	32.4	23.9	20.6	23.1	22.3
D segments	27.5	16.7	25.9	29.9	

germline D segment database. Only four alignments are above the 99% confidence threshold, providing little evidence for multiple D segments (Figure 5h).

Finally, to check for other germline elements that contribute to H3, sequences flanked by unmutated V_H and J_H segments that could not be confidently assigned to one of the major D segments were realigned to the complete nucleotide sequence of the D locus. From the 31 sequences examined, we were unable to identify any germline alignments above the 99% confidence threshold.

We conclude that the 27 major D segments on chromosome 14 (and their alleles) are the only germline elements that contribute to H3 diversity.

In addition, there is little evidence for D segment inversion or D-D recombination.

N-nucleotide addition creates significant H3 diversity

Any "extra" H3 sequence remaining after the confident assignment of V_H, D and J_H segments must be due to N or P-nucleotide addition (Alt & Baltimore, 1982; Lafaille *et al.*, 1989). Since P-nucleotide addition is not thought to occur at nucleolytically processed segment ends (Lafaille *et al.*, 1989) and more than 96% of our 893 somatically rearranged sequences do appear to be processed (data not shown), it seems likely that P-nucleotide

Table 2. D segment and reading frame use in 893 heavy chain sequences

% of 893 H3	Stop	%	Hydrophilic	%	Hydrophobic	%	
D1-1	0.56	VQLER	0	YNWND	60.0	GTTGT	40.0
D1-7	0.45	V*LEL	0	YNWNY	75.0	GITGT	25.0
D1-14	0	V*PEP	0	YNRNH	0	GITGT	0
D1-20	0.67	V*LER	0	YNWND	66.7	GITGT	33.3
D1-26	1.68	V*WELL	0	YSGSY	46.7	GIVGAT	53.3
D2-2	3.70	RIL**YQLLY	6.0	GYCSSTSCYT	66.7	DIVVVPAI	27.3
D2-8	0.78	RILY*WCMLY	0	GYCTNGVCYT	71.4	DIVLMVYAI	28.6
D2-15	2.35	RIL*WW*LLL	4.7	GYCSGGSCYS	66.7	DIVVVVAAT	28.6
D2-21	1.23	SILWW*LLF	0	AYCGGDCYS	54.6	HIVVVTAI	45.4
D3-3	4.82	VLRFLEWLLY	18.6	YYDFWSGYYT	58.1	ITIFGVVII	23.3
D3-9	2.13	VLRYFDWLL*	36.8	YYDILTGYYN	63.2	ITIF*LVII	0
D3-10	8.06	VLLWFGEILL*	11.1	YYYGSGSYNN	52.8	ITMVRGVVII	36.1
D3-16	1.01	VL*LRLGEELSLY	0	YYDYVWGSYRT	66.7	IMITFGGVIVI	33.3
D3-22	3.81	VLL***WLL	2.9	YYDSSGYYY	88.3	ITMIVVVIT	8.8
D4-4	0.34	*LQ*L	0	DYSNY	83.3	TTVT	16.7
D4-11	0.33	*LQ*L	0	DYSNY	83.3	TTVT	16.7
D4-17	2.35	*LR*L	0	DYGDY	61.9	TTVT	38.1
D4-23	1.12	*LRW*L	0	DYGGNS	50.0	TTVVT	50.0
D5-5	1.18	WIQLWL	4.8	GYSYGY	61.9	VDTAMV	33.3
D5-12	1.57	WI*WLRL	0	GYSGYDY	71.4	VDIVATI	28.6
D5-18	1.17	WIQLWL	4.8	GYSYGY	61.9	VDTAMV	33.3
D5-24	0.78	*RWLQL	0	RDGYN	57.1	VEMATI	42.9
D6-6	1.34	V*QLV	8.3	EYSSSS	33.4	SIAAR	58.3
D6-13	3.47	V*QQLV	8.8	GYSSSWY	61.3	GIAAG	29.0
D6-19	4.70	V*QWLV	11.9	GYSSGKY	42.9	GIAVAG	45.2
D6-25	0	V*QRL	0	GYSSGY	0	GIAAA	0
D7-27	0.90	*LG	0	NWG	25.0	LTG	75.0
No D	49.5						

The first column shows the percentage of the 893 somatically rearranged H3 sequences that can be confidently aligned to each D segment (alignments that exceed the 99% confidence threshold). For the pairs of identical D segments, D4-4/D4-11 and D5-5/D5-18, the percentages of H3 sequences that align to them are divided equally. The rest of the Table shows the amino acid translation of each reading frame of all 27 D segments in single-letter code. The percentage of H3 sequences using each reading frame of any given D segment with respect to J_H is shown alongside the amino acid translation. Stop codons are shown as asterisks (*). Amino acid sequences in bold type were identified amongst the 893 H3 sequences. Reading frames are defined according to the presence of stop codons, hydrophilic or hydrophobic amino acid residues (see the text). Note that for 49.5% of the 893 rearranged H3 sequences we were unable to confidently assign a D segment (No D).

addition plays a minor role in H3 diversification. In addition, the nucleotide composition of the extra H3 sequence (Table 1) is similar to that ascribed to N-nucleotide addition by terminal deoxynucleotidyl transferase (TdT; Basu *et al.*, 1983; Gauss & Lieber, 1996). In contrast to a recent report on N-nucleotide addition in T-cell receptor β chains (Rowen *et al.*, 1996), there is no difference between the nucleotide composition of N-nucleotides 5' and 3' of the D segment (Table 1), suggesting that in B-cells, TdT acts at all segment termini rather than predominantly from D segments as seems to be the case in T-cells.

Although some of the 442 H3 sequences for which a germline D segment could not be confidently assigned may be highly mutated versions of known D segments, most are G + C-rich, suggesting that they may be mainly generated by the activity of TdT (for comparison, Table 1 shows the nucleotide composition of the 27 germline D segments from chromosome 14). In these cases, the germline D segment may be acting purely as an anchor for considerable N-nucleotide diversification.

Use of different D segments and D segment reading frames

In total, 451 rearranged H3 sequences in our database can be confidently assigned to one of the 27 D segments on chromosome 14 (Figure 5g). The use of each D segment is summarised in Table 2. As noted in previous studies, the D3, D6 and D2 families predominate (Sanz, 1991; Yamada *et al.*, 1991; Huang *et al.*, 1992; Brezinschek *et al.*, 1995), with nine D segments (D2-2, D2-15, D3-3, D3-9, D3-10, D3-22, D4-17, D6-13 and D6-19) being used more often than would be expected at random. We note that two of the 27 D segments are not seen at all (D1-14 and D6-25): these segments have mutations within their heptamer sequences that would be expected to prevent or severely restrict recombination (Figure 2; Gellert, 1992; Akamatsu *et al.*, 1994). A similar mutation in the heptamer of D4-4 may also prevent its rearrangement (Figure 2), but since its coding sequence is the same as D4-11 it is impossible to determine which of the two segments is used.

For each D segment family there is one reading frame that tends to encode a stop codon, followed by a reading frame that tends to encode glycine residues in conjunction with polar/hydrophilic residues (especially tyrosine and serine), followed by a third reading frame that is normally hydrophobic in character (with many alanine, valine and isoleucine residues). Instead of defining each reading frame relative to the RSS (Ichihara *et al.*, 1988) we therefore define it according to its character (see Table 2). The frequency of use of the three reading frames for each D segment is given in Table 2, together with an amino acid translation of each frame. The presence of a stop codon does not prevent

use of this reading frame, as nucleotide loss during VDJ recombination may remove it. The hydrophilic reading frame predominates in those human H3 sequences for which a confident D segment assignment can be made (60% of sequences, Table 2). In contrast to most of the other segments that normally use the hydrophilic reading frame, the D segments D6-6 and D6-19 are most frequently seen in the hydrophobic reading frame (Table 2).

Discussion

By systematic comparison of the complete sequence of the human immunoglobulin D segment locus with a database of rearranged sequences, we found that DIR segments, inverted D segments, minor D segments or D-D joins produce alignments no better than those produced by randomly generated sequences. In marked contrast, we found overwhelming evidence for conventional VDJ recombination involving 25 of the 27 D segments on chromosome 14, as shown by the majority of alignments scoring higher than those produced by randomly generated sequences. A recent PCR-based study also failed to detect any DIR segment rearrangement in developing B-cells (Moore & Meek, 1995). In the absence of any real evidence, we therefore believe that it is unreasonable to propose a role for the use of DIR segments, inverted D segments, "minor" D segments or D-D joins in the human antibody repertoire.

We have also shown that the use of different D segments and D segment reading frames is highly biased (Table 2). It is an inevitable consequence of scoring sequence alignments according to length that shorter D segments will have fewer rearranged counterparts. Nevertheless, other factors clearly influence the pattern of D segment use, as there are numerous examples of short D segments (most notably D6-19 and D4-17) being seen more often than long D segments (see Table 2). In addition, the fact that the use of D3-10 is twice that of D3-3 (which have identical RSS and are the same length) and that of D2-2 is five times that of D2-8 (which also have identical RSS and length) indicates that structural or selective pressures acting at the amino acid level must affect D segment use. This is also probably the major factor that affects reading frame use (which is not subject to any length biases). The most popular reading frame of almost all human D segments (Table 2) frequently encodes serine, tyrosine and glycine residues. This amino acid composition is similar to that seen in the five other antigen binding loops (see V BASE), suggesting that these residues are particularly good at binding antigen and/or for recruiting somatic hypermutation (for a more in depth discussion of D segment use, see Corbett, 1996).

For many of the rearranged sequences it was impossible to identify a source for the nucleotides located between the end of the V_H segment and the

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Materials

Shotgun seq and COS24

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beginning of the J_H segment. On average, these $H3$ sequences are shorter (22.3 nucleotides, Table 1) than those for which D segments can be confidently assigned (29.6 nucleotides) and are probably the result of either extensive nucleotide loss from D segments together with N-nucleotide addition and/or high levels of somatic mutation (both of which are accepted mechanisms of diversification). Indeed, on the basis of their nucleotide composition "fingerprint" (Table 1) and the average length of N-nucleotide addition in $H3$ sequences where D segments have been assigned (13.6 nucleotides, Table 1), it appears that these sequences are mainly the result of TdT activity.

Despite the mechanistic constraints and the various biases described above, there is still more sequence and length diversity in the human $H3$ region than in any other species so far examined (Wu *et al.*, 1993). The mouse (Ichihara *et al.*, 1989; Feeney & Riblet, 1993), chicken (Reynaud *et al.*, 1991) and rabbit (Knight & Crane, 1994) all have fewer germline D segments and fewer families. Furthermore, their D segments are usually (>80%) expressed in the hydrophilic reading frame (Kaartinen & Makela, 1985; Gu *et al.*, 1991; Knight & Crane, 1994; Reynaud *et al.*, 1994), whereas in humans only 60% of antibodies use the hydrophilic reading frame (Table 2). In this way, the use of many different D segments and reading frames in humans by conventional VDJ recombination together with extensive N-nucleotide addition creates a wide range of $H3$ structures and hence a vast repertoire of antigen binding sites.

Materials and Methods

Shotgun sequencing of cosmids COS21, COS23 and COS24

The shotgun sequencing strategy was as described (Wilson *et al.*, 1994; Baxendale *et al.*, 1995). Briefly, cosmids were fragmented by sonication and randomly subcloned into M13mp18. Greater than sevenfold coverage was achieved by sequencing at least 600 single-stranded M13 DNA templates per cosmid. Single-stranded DNA was prepared from subclones using anti-M13 magnetic beads. Templates were sequenced using the dideoxy chain termination cycle sequencing technique with dye-labelled M13-specific forward primers. Products were then run on an Applied Biosystems 373A stretch-liner sequencer. The sequences of COS21 and COS24 were compiled and assembled with the Staden package (Staden, 1994), whilst COS23 was initially assembled using the phrap algorithm (P. G. Green, unpublished) and was then incorporated into Staden package format by phrap2gap (S. Dear, R. Durbin, R. Mott, G. Marth & D. Hodgson, unpublished) for further manipulation. Sequence problems were resolved using a combination of reverse reads, long reads, oligo walks and Taq terminators. The accuracy of the entire 92,588 bp sequenced is estimated to be 99.98% with the methodology used (Wilson *et al.*, 1994; Whitfield *et al.*, 1995). The EMBL data library accession number for the complete sequence is X97051.

Computer-aided sequence analysis

This was performed as described (Whitfield *et al.*, 1995). Briefly, completed sequence was analysed on a cosmid-by-cosmid basis. Each cosmid was screened for repetitive elements using: the human repeat sequence database assembled by J. Jurka available from the PYTHIA server (email: pythia@anl.gov); a hidden Markov model to identify *Alu* elements; BLASTN to identify L1 and MER/SINE sequences; the programs QUICK-TANDEM and TANDEM to identify tandem repeats. The subfamily allocation of *Alu* and L1 elements was performed using the programs ALUS and CENSOR from the PYTHIA server, respectively. Repetitive sequence was then masked with the character N before BLASTN ($B = 1,000,000$) and BLASTX ($B = 1,000,000$, $S = 50$, $M = \text{BLOSUM62-12}$) were used to screen the remaining non-repetitive sequence against the following databases to identify coding regions and other features of interest: EMBL release 39; SWIR version 8 (which is a Sanger Centre non-redundant compilation of SWISS-PROT31, PIR43 and WORMPEP8); dbEST version 3.5. No other significant potential coding region could be identified with GRAIL II or by searching for CpG islands with the program CPG.

Germline Query (GQ)

GQ is an AWK script that takes an antibody variable region sequence and uses the BLAST algorithm to determine the best germline V, D and J segment alignments in the V BASE directory of human immunoglobulin genes (available on the World Wide Web: <http://www.mrc-cpe.cam.ac.uk/imt-doc/vbase-home-page.html> or on disk from I.M.T.). The GQ alignment process is summarised in Figure 4. V_H and J_H alignments were accepted if they exceeded 50% and 85% homology, respectively. In addition, V_H alignments were rejected if they ended more than ten nucleotides before the 3' end of the germline segment sequence, and J_H segment alignments had to be a minimum length of 18 nucleotides. The boundaries of segment alignments were defined by a series of contiguous mismatches with boundary conditions set as seven, two and three nucleotides for the V_H , D and J_H regions, respectively. Alignment scores were calculated using a system of +5 for a match and -4 for a mismatch.

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References

Akamatsu, Y., Tsurushita, N., Nagawa, F., Matsuoka, M., Okazaki, K., Imai, M. & Sakano, H. (1994).

Essential residues in V(D)J recombination signals. *J. Immunol.* 153, 4520–4529.

Alt, F. W. & Baltimore, D. (1982). Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-J_H fusions. *Proc. Natl Acad. Sci. USA* 79, 4118–4122.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410.

Basu, M., Hegde, M. V. & Modak, M. J. (1983). Synthesis of compositionally unique DNA by terminal deoxynucleotidyl transferase. *Biochem. Biophys. Res. Commun.* 111, 1105–1112.

Baxendale, S., Abdulla, S., Elgar, G., Buck, D., Berks, M., Micklem, G., Durbin, R., Bates, G., Brenner, S., Beck, S. & Lehrach, H. (1995). Comparative sequence analysis of the human and pufferfish Huntingdon's disease genes. *Nature Genet.* 10, 67–76.

Brezinschek, H. P., Brezinschek, R. I. & Lipsky, P. E. (1995). Analysis of the heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction. *J. Immunol.* 155, 190–202.

Buluwela, L. & Rabbits, T. H. (1988). A V_H gene is located within 95 kb of the human immunoglobulin heavy chain constant region genes. *Eur. J. Immunol.* 18, 1843–1845.

Buluwela, L., Albertson, D. G., Sherrington, P., Rabbits, P. H., Spurr, N. & Rabbits, T. H. (1988). The use of chromosomal translocations to study human immunoglobulin gene organization: mapping D_H segments within 35 kb of the C_H gene and identification of a new D_H locus. *EMBO J.* 7, 2003–2010.

Cook, G. P. & Tomlinson, I. M. (1995). The human immunoglobulin V_H repertoire. *Immunol. Today* 16, 237–242.

Cook, G. P., Tomlinson, I. M., Walter, G., Riethman, H., Carter, N. P., Buluwela, L., Winter, G. & Rabbits, T. H. (1994). A map of the human immunoglobulin V_H locus completed by analysis of the telomeric region of chromosome 14q. *Nature Genet.* 7, 162–168.

Corbett, S. J. (1996). The human immunoglobulin diversity segment repertoire. PhD thesis, University of Cambridge, UK.

Feeley, A. J. & Riblet, R. (1993). D_{ST4}: a new and probably the last, functional D_H gene in the BALB/c mouse. *Immunogenetics*, 37, 217–221.

Gauss, G. H. & Lieber, M. R. (1996). Mechanistic constraints on diversity in human V(D)J recombination. *Mol. Cell. Biol.* 16, 258–269.

Gellert, M. (1992). Molecular analysis of V(D)J recombination. *Annu. Rev. Genet.* 22, 425–446.

Gu, H., Kitamura, D. & Rajewsky, K. (1991). B Cell development regulated by gene rearrangement: arrest of maturation by membrane-bound D_H protein and selection of D_H element reading frames. *Cell*, 65, 47–54.

Huang, C., Stewart, A. K., Schwartz, R. S. & Stollar, B. D. (1992). Immunoglobulin heavy chain gene expression in peripheral blood B lymphocytes. *J. Clin. Invest.* 89, 1331–1343.

Ichihara, Y., Matsuoka, H. & Kurosawa, Y. (1988). Organization of human immunoglobulin heavy chain diversity gene loci. *EMBO J.* 7, 4141–4150.

Ichihara, Y., Hayashida, H., Miyazawa, S. & Kurosawa, Y. (1989). Only D_{FL16}, D_{SP2}, and D_{Q52} gene families exist in mouse immunoglobulin heavy chain diversity loci, of which D_{FL16} and D_{SP2} originate from the same primordial D_H gene. *Eur. J. Immunol.* 19, 1849–1854.

Kaartinen, M. & Makela, O. (1985). Reading of D genes in variable frames as a source of antibody diversity. *Immunol. Today* 6, 324–327.

Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S. & Foeller, C. (1991). *Sequences of Proteins of Immunological Interest*, vols 1, 2 and 3, U.S. Department of Health and Human Services.

Knight, K. L. & Crane, M. A. (1994). Generating the antibody repertoire in rabbit. *Advan. Immunol.* 56, 179–218.

Komori, T., Okada, A., Stewart, V. & Alt, F. W. (1993). Lack of N regions in antigen receptor variable region genes of TdT-deficient lymphocytes. *Science*, 261, 1171–1175.

Korenberg, J. R. & Rykowski, M. C. (1988). Human genome organisation: Alu, Lines, and the molecular structure of metaphase chromosome bands. *Cell*, 53, 391–400.

Lafaille, J. J., DeCloux, A., Bonneville, M., Takagaki, Y. & Tonegawa, S. (1989). Junctional sequences of T cell receptor gamma delta genes: implications for gamma delta T cell lineages and for a novel intermediate of V-(D)-J joining. *Cell*, 59, 859–870.

Legouis, R., Hardelin, J.-P., Levilliers, J., Claverie, J.-M., Compain, S., Wunderle, V., Millasseau, P., Le Paslier, D., Cohen, D., Caterina, D., Bougueret, L., Delemarre-Van de Waal, H., Lutfalla, G., Weissenbach, J. & Petit, C. (1991). The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. *Cell*, 67, 423–435.

Matsuda, F., Lee, K. H., Nakai, S., Sato, T., Kodaira, M., Zong, S. Q., Ohno, H., Fukuhara, S. & Honjo, T. (1988). Dispersed localization of D segments in the human immunoglobulin heavy-chain locus. *EMBO J.* 7, 1047–1051.

Matsuda, F., Shin, E. K., Hirabayashi, Y., Nagaoka, H., Yoshida, M. C., Zong, S. Q. & Honjo, T. (1990). Organization of variable region segments of the human immunoglobulin heavy chain: duplication of the D5 cluster within the locus and interchromosomal translocation of variable region segments. *EMBO J.* 9, 2501–2506.

Moore, B. B. & Meek, K. (1995). Recombination potential of the human DIR elements. *J. Immunol.* 154, 2175–2187.

Mortari, F., Wang, J.-Y. & Schroeder, H. W., Jr (1993). Human cord blood antibody repertoire. *J. Immunol.* 150, 1348–1357.

Nagaoka, H., Ozawa, K., Matsuda, F., Hayashida, H., Matsumura, R., Haino, M., Shin, E. K., Fukita, Y., Imai, T., Anand, R., Yokoyama, K., Eki, T., Soeda, E. & Honjo, T. (1994). Recent translocation of variable and diversity segments of the human immunoglobulin heavy chain from chromosome 14 to chromosomes 15 and 16. *Genomics*, 22, 189–197.

Ravetch, J. V., Siebenlist, U., Korsmeyer, S., Waldmann, T. & Leder, P. (1981). Structure of the human immunoglobulin μ locus: characterization of embryonic and rearranged J and D genes. *Cell*, 27, 583–591.

Reynaud, C.-A., Anquez, V. & Weill, J.-C. (1991). The chicken D locus and its contribution to the immunoglobulin heavy chain repertoire. *Eur. J. Immunol.* 21, 2661–2670.

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Reynaud, C.-A., Bertocci, B., Dahan, A. & Weill, J.-C. (1994). Formation of the chicken B-cell repertoire: ontogeny, regulation of Ig gene rearrangement, and diversification by gene conversion. *Advan. Immunol.* 57, 353-378.

Rowen, L., Koop, B. F. & Hood, L. (1996). The complete 685-kilobase DNA sequence of the human β T cell receptor locus. *Science*, 272, 1755-1762.

Sanz, I. (1991). Multiple mechanisms participate in the generation of diversity of human H chain CDR3 regions. *J. Immunol.* 147, 1720-1729.

Sanz, I., Wang, S.-S., Meneses, G. & Fischbach, M. (1994). Molecular characterization of human Ig heavy chain DIR genes. *J. Immunol.* 152, 3958-3969.

Shin, E. K., Matsuda, F., Nagaoka, H., Fukita, Y., Imai, T., Yokoyama, K., Soeda, E. & Honjo, T. (1991). Physical map of the 3' region of the human immunoglobulin heavy chain locus: clustering of antibody-related variable segments in one haplotype. *EMBO J.* 10, 3641-3645.

Siebenlist, U., Ravetch, J. V., Korsmeyer, S., Waldmann, T. & Leder, P. (1981). Human immunoglobulin D segments encoded in tandem multigenic families. *Nature*, 294, 631-635.

Sonnhammer, E. L. L. & Durbin, R. (1995). A dot-matrix program with dynamic threshold control suited for genomic DNA and protein sequence analysis. *Gene*, 167, GC1-10.

Staden, R. (1994). The Staden package. In *Methods in Molecular Biology* (Griffin, A. M. & Griffin, H. G., eds), pp. 9-170, Humana Press Inc., Totowa, USA.

Tomlinson, I. M., Walter, G., Marks, J. D., Llewelyn, M. B. & Winter, G. (1992). The repertoire of human germline V_H sequences reveals about fifty groups of V_H segments with different hypervariable loops. *J. Mol. Biol.* 227, 776-798.

Tomlinson, I. M., Cook, G. P., Carter, N. P., Elaswarapu, R., Smith, S., Walter, G., Buluwela, L., Rabbits, T. H. & Winter, G. (1994). Human immunoglobulin V_H and D segments on chromosomes 15q11.2 and 16p11.2. *Hum. Mol. Genet.* 3, 853-860.

Tomlinson, I. M., Walter, G., Jones, P. T., Dear, P. H., Sonnhammer, E. L. L. & Winter, G. (1996). The imprint of somatic hypermutation on the repertoire of human germline V genes. *J. Mol. Biol.* 256, 813-817.

Tonegawa, S. (1983). Somatic generation of antibody diversity. *Nature*, 302, 575-581.

Tuailon, N., Miller, A. B., Tucker, P. W. & Capra, J. D. (1995). Analysis of direct and inverted D_{H1} rearrangements in a human Ig heavy chain transgenic minilocus. *J. Immunol.* 154, 6453-6465.

Whitfield, L. S., Hawkins, T. L., Goodfellow, P. N. & Sulston, J. (1995). 41 kilobases of analyzed sequence from the pseudoautosomal and sex-determining regions of the short arm of the human Y chromosome. *Genomics*, 27, 306-311.

Wilson, I. A. & Stanfield, R. L. (1994). Antibody-antigen interactions: new structures and new conformational changes. *Curr. Opin. Struct. Biol.* 4, 857-867.

Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T. & Cooper, J., et al. (1994). 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans*. *Nature*, 368, 32-38.

Wu, T. T., Johnson, G. & Kabat, E. A. (1993). Length distribution of CDRH3 in antibodies. *Proteins: Struct. Funct. Genet.* 16, 1-7.

Yamada, M., Wasserman, R., Reichard, B. A., Shane, S., Caton, A. J. & Rovera, G. (1991). Preferential utilization of specific immunoglobulin heavy chain diversity and joining segments in adult human peripheral blood B lymphocytes. *J. Exp. Med.* 173, 395-407.

Zietkiewicz, E., Richer, C., Makalowski, W., Jurka, J. & Labuda, D. (1994). A young Alu subfamily amplified independently in human and African great apes lineages. *Nucl. Acids Res.* 22, 5608-5612.

Zong, S. Q., Nakai, S., Matsuda, F., Lee, K. H. & Honjo, T. (1988). Human immunoglobulin D segments: isolation of a new D segment and polymorphic deletion of the D_1 segment. *Immunol. Letters*, 17, 329-334.

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